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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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R C van Dijk

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Hybrid proteins of beta-lactamase class A

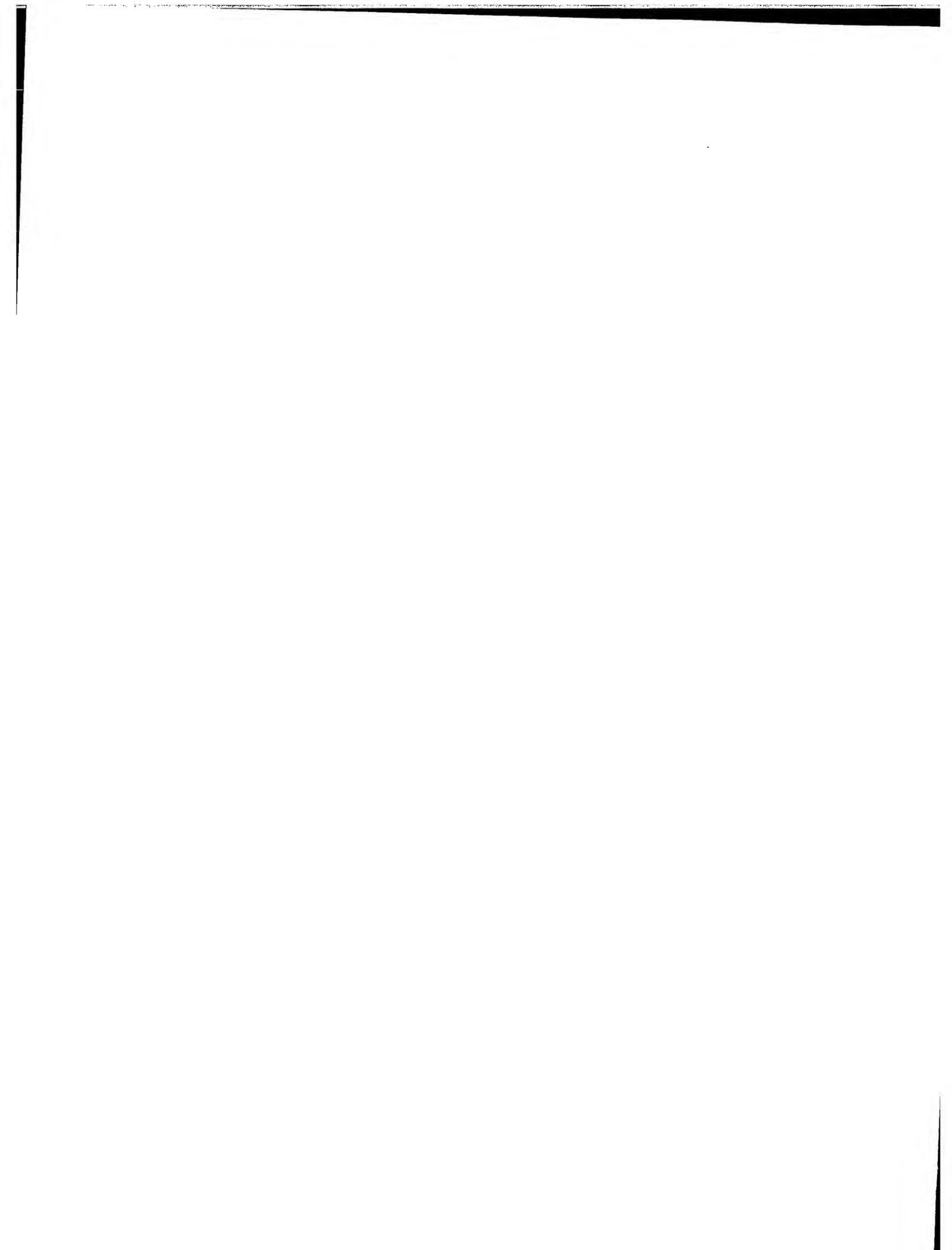
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Hybrid proteins of β -lactamase class A

(67)

[0001] The present invention relates to hybrid proteins of β -lactamase class A.

[0002] In the state of art a plurality of scientific publications have described the construction of fusion proteins. In the majority of these cases, such fusion proteins were realised as fusions of polypeptides to the N-terminus or C-terminus of a carrier protein. Martineau et al. (Martineau, P., J. G. Guillet, et al. (1992). "Expression of heterologous peptides at two permissive sites of the MalE protein: antigenicity and immunogenicity of foreign B-cell and T-cell epitopes." Gene 118(1): 151; Martineau, P., C. Leclerc, et al. (1996). "Modulating the immunological properties of a linear B-cell epitope by insertion into permissive sites of the MalE protein." Mol Immunol 33(17-18): 1345-58.) have realized the insertion of a protein into permissive sites of the protein MalE and they have studied the immune response against the inserted protein.

[0003] Several authors describe the immunisation against the enterotoxins STa (heat stable enterotoxin of *E. coli*) via constructing N-terminal or C-terminal fusion proteins, wherein different carrier proteins are involved, in order to obtain an immune response against the STa peptide, which as such is not immunogenic. The construction of hybrid proteins by inserting the STa peptide into a permissive site of a carrier protein has not yet been described in the prior art. The β -lactamase TEM-1 also not has been used as carrier protein for the construction of hybrid or fusion proteins with STa.

[0004] The process for synthesising bifunctional proteins got underway through binding one protein to another by chemical means. Against the background of this approach, the two proteins of interest, which have different properties, are synthesised independently and then treated with a chemical so as to achieve the covalent bonding of specific chemical groups available in the proteins. The technique has helped to achieve progress in developing diagnosis tests and recombinant vaccines. However, it has several drawbacks that have prompted the scientific community to develop other options. One of the disadvantages of using chemical agents is the aspecific coupling of two target proteins, which results in a lack of uniformity in the way the two proteins are bad associated and oriented. This in turn

may inactivate one of the proteins. As a result of the binding system, protein complexes are formed where the stoichiometry and composition is heterogeneous. For example, a protein X may be associated with one, two, three or more Y proteins. A binding between identical molecules (dimer-multimer) is difficult to avoid, thereby reducing the quantity of bifunctional proteins obtained. The resulting assay challenges can be intuitively understood, along with the calibration stage required to assess the sensitivity of the product with each further coupling.

[0005] Another challenge with the aspecific binding of the bridging agents is that these techniques call for large quantities of proteins in return for a reduced yield potential, thereby pushing up the costs of the finished product. A de novo synthesis of bifunctional proteins in prokaryotics or eukaryotics systems offers an alternative way of meeting these challenges. This method involves molecular biology techniques providing an opportunity to modify the structure of the coding gene for the proteins in question. However, this technology calls for a detailed knowledge of the biochemical and structural properties of the polypeptides synthesised on the basis of their manipulated sequence of nucleotides.

[0006] An initial de novo synthesis approach was adopted on the basis of fusion proteins. This involves genetically fusing coding DNA sequences for two proteins of interest to one or the other ends of the said genes. This fusion operation may apply to whole proteins, fragments of proteins or random peptides. The two proteins (or protein fragments) are then expressed in tandem by the producer organism. This technique solves the problems of difficult, insoluble, misfolding proteins. It also addresses issues related to chemical coupling (see above), even though it is not the technique by which other ones are judged in this sphere. Presenting a peptide to the ends of another protein means this protein is exposed to excessive proteolysis when the fusion protein is being produced and purified. What is more, the degrees of freedom of the fusion peptides are such that they seriously destabilise the structure of the entire fusion. The result is a total loss of biological activity.

[0007] Therefore it was object of the present invention to provide functional proteins wherein the respective carrier protein retains its activity and also the added heterologous sequence still processes its function (for example as epitope, enzyme

etc.), wherein furthermore the added heterologous sequence is somehow exposed on the surface of the carrier protein which is providing the possibility that the heterologous sequence may interact with other molecules. Furthermore, it was the object of the present invention to provide the functional proteins wherein the additional heterologous sequence maintains its free dimensional structure. It was a further object that the heterologous sequence is made less susceptible to proteolysis.

[0008] The object of the present invention is solved by a recombinant nucleotide acid sequence which codes upon expression for at least a part of a hybrid β -lactamase class A protein, wherein the β -lactamase class A protein is bearing at least one heterologous sequence in a region located between two neighbouring alpha helices of the β -lactamase sequence, wherein the region is selected from:

- a) the region forming a juncture between alpha helix 8 and alpha helix 9 of the TEM-1 β -lactamase;
- b) the region forming a juncture between the alpha helices of homologous β -lactamases class A, said alpha helices corresponding to the alpha helix 8 and alpha helix 9 of the TEM-1 β -lactamase.

[0009] In a preferred embodiment the hybrid β -lactamase is possessing an activity selected from

- a) hydrolysing β -lactams;
- b) binding covalently and in a stable manner to derivatives of β -lactams and inhibitors of β -lactamases.

[0010] Furthermore it is preferred that the hybrid β -lactamase retains its activity of hydrolysing β -lactams at least partially.

[0011] **Detailed Description of the Invention**

[0012] The technological advance offered by the present invention involves internalising the proteins or polypeptide fragments within the native structure of a carrier protein, a β -lactamase. This new approach provides a means of replacing the

internalised fragments in a 3D context close to the native situation. The constraints of the carrier protein imposes compel the internalised peptides to adopt a proper structure. This guarantees continuing biological activities in many cases. The outcome is the creation of a bifunctional hybrid protein. The hybrid β -lactamase according to the invention represents a single polypeptide. The use of this hybrid β -lactamase preferably is to study the interaction of the internalised homologous sequence with other separate molecules (for example antibodies) or to use this interaction of the internalised homologous sequence with other separate molecules in assays, either as binding interaction to be measured or as part of the test.

[0013] The catalytic efficiency and the plasticity of the class A β -lactamases means they are effective candidates for constructing bifunctional proteins as a result of inserting sequences of exogenous peptides within the enzyme structure.

[0014] The TEM-1 β -lactamase is an active class A serine enzyme involved in the bacterial resistance to β -lactam based antibiotics, such as penicillin and cephalosporins. The mature form of the TEM-1 β -lactamase is a monomeric protein with 263 amino acids. Its 3D structure is hallmarked by two areas one of which is helice-rich and the other comprises α helices and β sheets (figure 1).

[0015] The class A β -lactamases interact with the β -lactam based antibiotics to form an intermediate called acyl-enzyme where the antibiotic is covalently linked to the serine of the enzyme's active site (figure 2). The β -lactamases efficiently catalyse the deacylation stage (k_3). This regenerates the active enzyme and releases a biologically inactive substance, where the amide linkage of the β -lactam of the antibiotic's nucleus is hydrolysed. In the case of the TEM-1 β -lactamase mutagenesis, experiments have shown that the deacylation reaction could be inhibited to produce a stable acyl-enzyme complex by replacing the glutamate 166 residues in asparagine (6) (figure 2). This feature permits the immobilisation of TEM-1 β -lactamase hybrid protein on β -lactam coated matrix.

[0016] As a result of the specificity, catalytic efficiency and plasticity of the TEM-1 β -lactamase, this protein is a valuable enzyme for developing new processes for detecting, assaying and orienting peptides and proteins used for therapeutic

purposes. Towards this end, chromogenic and fluorescents substrates, suicide inhibitors and prodrug cytotoxic agents have been developed.

[0017] Transposition and phage display experiments have shown the possibility of introducing or degenerating very short sequences of nucleotides (8 to 30 nucleotides) in the coding sequence for the TEM-1 β -lactamase and using these mutated genes as a basis for synthesizing a constantly functional enzyme (3, 5, 14, 15). In the context of this technology (WO98/23731) phage libraries (10^{10} phages) bearing chimeras of the TEM-1 β -lactamase, where small degenerated peptides have been inserted, are used to select the degenerated peptide that has an affinity for a given target. This is a cumbersome, painstaking and evolutionary method, as it is not common for a peptide having a high affinity for the target to be selected. This characteristic involves creating several mutagenesis stages in respect of the peptide and the carrier protein so as to optimise the affinity of the hybrid protein. Hence this entails creating new banks and producing new screenings.

[0018] The idea of the present invention is not to create a chimera bank that theoretically covers all types of biodiversity but favours the insertion of large peptidic sequences present in proteins whose biochemical characteristics have already been clearly identified. Consequently, the present invention provides a means of internalising a peptidic sequence that has already been naturally optimised for a given property and averting (as is the case with the phage display technique) evolutionary mutagenesis reactions within the insert and carrier protein. With this system, the chimera banks may be restricted to a few thousand clones (or a few dozens) so the screening is quicker and more targeted.

[0019] Unlike the results obtained with the various TEM-1 β -lactamase utilisation methods, wherein the insertion site leads to a change in the TEM-1 enzymatic properties, this present invention marks a new departure because the inventors succeeded in identifying and creating, in a loop diametrically opposed to the active enzyme site (figure 1, site B: region Thr195 to Leu199), a region that is particularly favourable for internalising large exogenous sequences of peptides with a length preferably 11 or more amino acids. In this application this loop Thr195 to Leu199 is also referred to as the region forming the juncture between alpha helix 8 and alpha helix 9 of the TEM-1 β -lactamase and homologous enzymes which have a

homologous three-dimensional structure, for example like BlaP and BlaL. The results show that the polypeptides internalised in the context of this loop are able to adopt a folding close to their native conformation, as their biological activity is retained. This specific feature offers new prospects for constructing and using new generations of hybrid bifunctional proteins where a specific and effective enzymatic activity is associated with the biochemical properties of another protein or a fragment of a protein. This special feature is the second focal point of this invention, as it is of crucial importance for developing the diagnosis test or the chromatography affinity system, because the β -lactamase activity is used to quantify the protein-macromolecule interaction or to immobilise the hybrid protein in an oriented way.

[0020] As described in the patent application WO 98/23731 the TEM-1 enzyme was shown to be sensitive to the action of proteases during production in bacterial system, which reduces the output of hybrid proteins. By applying the technology according to the present invention also to β -lactamases that are more resistant to proteases (BlaP and BlaL) further advantageous constructs have been provided. These BlaP and BlaL β -lactamases are produced by Gram positive organisms for which suitable production tools exist. Furthermore, these bacteria are well-known in industry and enjoy GRAS (Generally Regarded As Safe) status. In the case of BlaP and BlaL it was possible to show that polypeptide insertion technique could be transferred to other β -lactamases of the same class and make a generally well-known improvement to the technology via the properties specific to these enzymes.

[0021] In a further preferred embodiment the nucleotide sequence coding for the β -lactamase sequence is selected from:

- a) nucleotide sequence coding for the β -lactamase TEM-1 (SEQ ID NO: 1; complementary strand);
- b) nucleotide sequence coding for the β -lactamase BlaP (SEQ ID NO: 2);
- c) nucleotide sequence coding for the β -lactamase BlaL (SEQ ID NO: 3);
- d) nucleotide sequences which hybridise under stringent conditions to the nucleotide sequences of any one of a), b) or c) or fragments thereof. It should be noted that the nucleotide sequence SEQ IDNO: 1 given for the β -lactamase TEM-1 here is the complementary strand.

[0022] In a further preferred embodiment the heterologous sequence is partially or fully replacing the region between alpha helix 8 and alpha helix 9 or the region between alpha helix 9 and alpha helix 10. As described above the numbering of the helices refers to the TEM-1 β -lactamase. The present invention also includes homologous β -lactamases of class A, so that the identification of helix 8 and helix 9 in those homologous enzymes has to be applied in a corresponding manner. In case of the TEM-1 β -lactamase this region is defined by the amino acid residues Thr195 – Leu199.

[0023] In a further preferred embodiment the heterologous sequence has a length of 11 or more amino acid residues, preferably in the range of 11 to 5000 amino acid residues, more preferably in the range of 11 to 3000 amino acid residues, more preferred in the range of 11 to 2000 amino acid residues, and further preferred in the range of 11 to 1000, even more preferred in the range of 11 to 300, and most preferred in the range of 18 to 200 amino acid residues. According to the present invention the insertion site within the β -lactamase class A for the homologous sequence is determined in that way that any sequence of any length can be inserted essentially without disturbing the three-dimensional structure and the activity of the β -lactamase (also the so called "carrier protein"), as shown below.

[0024] In a further preferred embodiment the hybrid β -lactamase is a bifunctional protein. Since the carrier protein, the β -lactamase moiety of the hybrid β -lactamase retains its activity and also the heterologous sequence originally also has a kind of function (for example as an epitope) the hybrid β -lactamase possesses two functions and therefore is a bifunctional protein.

[0025] In a further preferred embodiment the heterologous sequence is related to a function. In particularly preferred embodiments the function of the heterologous sequence as such is selected from: being an epitope, being a specific binding partner for antibodies, being specially recognised and bound by antibodies, having a binding affinity to earth alkaline ions and metal ions, having enzymatic activity, being a toxin (for example STa heat-stable enterotoxin of *E. coli*), bearing a glycosylation site, bearing a glycosylated peptide, being a specific binding partner for any polypeptide or any ligand, having a binding affinity to dsDNA and ssDNA or RNA (having a binding affinity to nucleotide and polynucleotide).

[0026] Furthermore, it is particularly preferred that the heterologous sequence is selected from the group: STa (heat stable enterotoxin of *Escherichia coli*, SEQ ID NO: 21), protein A of *Staphylococcus aureus*, (SEQ ID NO: 23 and 25), protein G of *Streptococcus pyogenes*, (SEQ ID NO: 27 and 29), a linear antigenic determinant of the hemagglutinin of the Influenca virus (SEQ ID NO: 31), a fragment of human phospholipase – type II (hPLA₂) (SEQ ID NO: 33), LPS binding amino acid sequence (SEQ ID NO: 35), and nucleotide sequences which hybridise under stringent conditions to said nucleotide sequences or fragments thereof.

[0027] Furthermore the present invention provides a recombinant polypeptide which is encoded by the recombinant nucleotide sequence as described before. The present invention therefore provides a recombinant polypeptide comprising at least a part of a β-lactamase class A protein, wherein that the β-lactamase class A protein is bearing at least one heterologous sequence in a region located between two neighboring alpha helices of the β-lactamase sequence, wherein the region is selected from:

- a) the region forming a juncture between alpha helix 8 and alpha helix 9 of the TEM-1 β-lactamase;
- b) the region forming a juncture between the alpha helices, which correspond to the alpha helix 8 and alpha helix 9 of the TEM-1 β-lactamase.

[0028] The further preferred embodiments of the recombinant polypeptide is outlined in respect to the description of the respective nucleotide sequence endocding the hybrid β-lactamase.

[0029] Furthermore, the present invention provides the use of the recombinant nucleotide sequence of the recombinant polypeptide for vaccination. As described above the enterotoxin of *Escherichia coli* (STa) as such is not immunogenic. However, by incorporating this peptide into a β-lactamase class A protein, namely in a region on the surface of this carrier protein, the possibility is given to raise antibodies against the heterologous sequence (STa), as shown below. Therefore, it is also preferred to use the recombinant nucleotide sequence or the recombinant polypeptide of the present invention for raising antibodies against the heterologous sequence. A further preferred embodiment is the use of the same for epitope mapping for a different protein or polypeptide. For epitope mapping smaller peptides having for example a length of 5 to 30 amino acid residues which are covering the

polypeptide to be examined are used for being introduced as heterologous sequence in the region forming a juncture between alpha helix 8 and alpha helix 9 of the TEM-1 β -lactamase or homologs thereof. As result a set of hybrid β -lactamase class A protein is constructed bearing (overlapping) sequences as heterologous sequence of the polypeptide to be examined. This set of hybrid β -lactamases differing within the homologous sequence is then used for studying the epitopes of the polypeptide in question can be studied.

[0030] The present invention also provides the use of the recombinant nucleotide sequence or the recombinant polypeptide of the present invention for affinity chromatography, particularly for the concentration and/or purification of antibodies directed against the heterologous sequence of the hybrid β -lactamase. By using a mutant of the β -lactamase class A protein (glutamate 166 asparagine) the β -lactamase can be immobilised on a matrix which is coated with the substrate (β -lactam). Since the hybrid β -lactamase is presenting the heterologous sequence on its surface it is possible to concentrate and/or purify antibodies which are directed against this heterologous sequence, which is immobilised on for example a column via the β -lactamase carrier protein, which is linked to its substrate on the matrix of the column.

[0031] In a similar way it is possible to detect molecules which are binding to the heterologous sequence qualitatively and/or quantitatively. It is particularly preferred that the molecules binding to the heterologous sequence are antibodies or antibody fragments, polypeptides, dsDNA, ssDNA, RNA or small ligands. The method which may apply for the qualitative and/or quantitative detection is well known as ELISA.

[0032] Furthermore the recombinant polypeptide preferably may be used in molecular diagnostics. For example, the protein A of *Staphylococcus aureus* or protein G of *Streptococcus pyogenes* or fragments thereof may be used as heterologous sequence incorporated into β -lactamase A protein. This heterologous sequence is exposed on the surface of the carrier protein (β -lactamase class A) and binds to the Fc region of antibodies. By using substrates of the β -lactamase which upon cleavage show a colour change, this system can be applied for the quantitative and/or qualitative detection of antigens to which the antibody is directed.

[0033] The present invention also provides a pharmaceutical composition comprising a recombinant polypeptide. For example a recombinant polypeptide of the present invention may be used for drug-targeting. The homologous sequence which is incorporated into the hybrid β -lactamase protein may be specifically selected from those determinants which are bound by cellular receptors (for example of cancerous cells or cells infected by a virus). Preferably a (therapeutically) inactive pro-drug is used, which is activated through cleavage by the β -lactamase moiety into an active drug. Then such cellular targets which are involved in a disease can be inhibited or destroyed. The present invention also provides the use of a recombinant polypeptide for the manufacture of a medicament for the preventive and/or therapeutic treatment of diseases selected from the group cancer, viral diseases and bacterial diseases (or infection diseases), autoimmune diseases and allergy.

[0034] The present invention also provides a method for screening a compound for treatment, prevention and/or diagnosis of a disease which comprises the step of detecting the interaction between the homologous sequence of the hybrid β -lactamase according to the present invention and a protein or polypeptide which binds to the homologous sequence in the presence of a compound to be tested. Preferably the compound to be tested is selected as the candidate of an effective medicament wherein the compound has an effect on the interaction between the homologous sequence inserted into the hybrid β -lactamase and the peptide which binds to the homologous sequence.

[0035] In a particularly preferred embodiment the method comprises the steps of:

- a) subjecting the recombinant polypeptide of the present invention and a polypeptide which binds to the homologous sequence to interaction with each other in the presence of the compound to be tested;
- b) subjecting the recombinant polypeptide of the present invention and a polypeptide which binds to the homologous sequence to interaction with each other in the absence of the compound to be tested;
- c) detecting the interactions in the steps a) and b), and
- d) comparing the interactions in the steps a) and b) to chose the compound having an effect on the interaction as a candidate of an effective medicament.

[0036] The present invention further provides a biological sensor comprising a recombinant polypeptide of the present invention. The term biosensor has been applied to devices either (1) used to monitor living systems, or (2) incorporate biologic or biomimetic elements. The consensus, however, is that the term should be reserved for use in the context of a sensor incorporating a biological element such as an enzyme, antibody, nucleic acid, microorganism or cell. The term "biosensor" as used in this patent application will be defined as:

analytical devices incorporating a biological material or a biomimetic material (e.g. tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids etc.), intimately associated with or integrated within a physicochemical transducer or transducing (micro)system, which may be optical, electrochemical, thermometric, piezoelectric or magnetic.

[0037] The usual aim of a biosensor is to produce either discrete or continuous digital electronic signals which are proportional to a single analyte or a related group of analytes.

[0038] In a preferred embodiment antibodies are immobilised on a conductive polymeric material. The hybrid protein carrying as homologous sequence an epitope which is specifically recognized and bound by the antibody is used for detecting the respective antibody. Upon cleavage of the substrate by the β -lactamase moiety protons will be generated which can be detected by potentiometric measurement. In an alternative embodiment the antigen is immobilised on the conductive polymeric material. By the use of a hybrid β -lactamase class A protein, wherein the heterologous sequence is binding to the Fc region of antibodies, the presence of antibodies directed to the immobilised antigen can be measured upon cleavage of the substrate and the generation of protons which again are detected by potentiometric measurement.

[0039] In a bid to validate the various fields of application, several hybrid proteins were constructed by inserting restriction sites in the DNA sequences of the TEM-1 β -lactamases, BlaP and BlaL. Belonging to the class A group of β -lactamases, these enzymes originate from *Salmonella typhimurium*, *Bacillus*

licheniformis and *Streptomyces cacaoi* respectively. The restriction sites were inserted in a region common to class A β -lactamases, so as to be able to retain β -lactamase activity after large exogenous sequences have been internalised.

[0040] Various exogenous nucleotide sequences were internalised in these recombinant genes. The hybrid genes produced during these operations provide a means of producing various bifunctional proteins, for example:

1. A hybrid protein of the TEM-1 β -lactamase where the STa heat stable enterotoxin protein of *Escherichia coli* is internalised (TEMSTA).
2. Hybrid proteins of the TEM-1 β -lactamase where 1 to 3 repeated domains of the *Staphylococcus aureus* protein A are internalised (TEM-PA).
3. Hybrid proteins of the BlaP β -lactamase where 1 to 3 repeated domains of the *Staphylococcus aureus* protein A are internalised (BlaP-PA).
4. Hybrid proteins of the TEM-1 β -lactamase where the domain/domains B1 and/or B2 of the protein G of *Streptococcus pyogenes* are internalised (TEM-PG).
5. Hybrid proteins of the BlaP β -lactamase where the domain/domains B1 and/or B2 of the protein G of *Streptococcus pyogenes* are internalised (BlaP-PG).
6. A hybrid protein of the BlaP β -lactamase where a linear antigenic determinant of the hemagglutinin of the Influenza virus is internalised (BlaP-HA).
7. Hybrid proteins of the TEM-1 β -lactamase where fragments of human phospholipase - type II, hPLA₂ are internalised (TEM-PLA2).
8. A hybrid protein of the BlaP β -lactamase where fragments of multimerised polypeptides comprising three amino acids repeated in tandem and presenting an affinity for bacterial endotoxins are internalised (BlaP-LPS).
9. Use of the hybrid protein BlaP-PA in an electrobiochemical biosensor system.

[0041] **Description of the figures**

Figure 1 shows the 3D structure of TEM-1 β -lactamase. A: active site of the enzyme. B: highly tolerant position to exogenous polypeptide insertion.

Figure 2 shows the model of β -lactamase hydrolysis of penicillin substrate.

Figure 3 shows the sequence of restriction cassettes internalised in TEM-1 coding sequence.

Figure 4 shows the 3D structures of TEM-1 and BlaP β -lactamases. Arrows show the polypeptide insertion site.

Figure 5 shows the sequence of *Sma*I and *Eco*RV restriction site introduced in BlaP and BlaL coding sequence, respectively.

Figure 6 shows the fold of the TEM-1 β -lactamase. The position of the permissive sites (filled square), the semi-permissive site (open square) and the non-permissive sites (grey square) are indicated.

Figure 7 shows the toxicity titration curve of the hybrid proteins.

Figure 8 shows the immunogenicity determined by ELISA for the TEM197H and TEM197STa. A) The presence of anti-TEM antibodies was estimated by coating 250 ng of TEM per well. B) The presence of anti-STa antibodies was estimated by coating 250 ng of GST-STa per well. The serum was diluted 100 fold in PBS buffer. The numbers below the columns of the diagram indicate different mouse individuals.

Figure 9 shows the titration curve of the anti-TEM IgG in the serum collected at day 56.

Figure 10 shows the isotopic response against the carrier protein (TEM197H). The numbers below the columns of the diagram indicate different mouse individuals.

Figure 11 shows the determination of the level of the anti-TEM IgG raised against TEM197H (1), TEM197STa (2), TEM216STa (3), TEM232STa (4)and TEM260STa (5). The numbers below the columns of the diagram indicate different mouse individuals.

Figure 12 shows the construction of hybrid proteins of the TEM-1 β -lactamase wherein one or more repeated domains of the *Staphylococcus aureus* protein A (figure 13 A) are internalised. A: protein A of *Staphylococcus aureus* is composed of five

repeated domains indicated by letters E, D, A, B and C. These domains bind the antibody Fc region. The sequence N-terminal of the E domain is the signal sequence (S). The sequence at the C-terminus is the peptidoglycan fixation domain (P). B shows the structure of the E domain. Each of the repeated domains of protein A is organised into three α helices. C: the DNA coding for the repeated domains of protein A was amplified by PCR. D: the agarose gel is showing restriction analysis of different hybrid β -lactamase clones bearing 1, 2 or 3 domains of protein A. E: the SDS-PAGE gel analysis shows the hybrid β -lactamase proteins wherein one or three domains of protein A have been incorporated.

Figure 13 shows the titration curve of immobilised rabbit IgG by TEM-PA hybrid protein. The adsorbance is plotted against the amount of fixed rabbit IgG (ng).

Figure 14 shows the construction of the hybrid proteins of the TEM-1 β -lactamase where the B1 and/or B2 domain or domains of the *Streptococcus pyogenes* protein G were internalised. A shows that protein G is composed of 2 repeated domains, called B1 and B2 that bind to the antibody Fc region. They confer an affinity for the antibodies Fc region. S is the signal peptide sequence of protein G. B shows that each of the 2 domains is organised with a β -sheet and α -helices. C shows that the nucleotide sequence encoding for the repeated domains of the G protein were cloned into the TEM-1 β -lactamase sequence. D shows an SDS-PAGE of hybrid β -lactamase TEM-1 having 2 domains of protein G internalised.

Figure 15 shows the nucleotide sequence of insertion site of BlaP β -lactamase and BlaP-HA hybrid protein.

Figure 16 shows a 12% SDS-PAGE gel electrophoresis of the BlaP and BlaP-HA β -lactamases after SFF partial purification of periplasmic fractions coming from *E. coli* strain transformed with pROGENO-1 BlaP(211/Smal) and pROGENO-1 BlaP-HA. Transformed bacteria were grown over night on rich medium at 37°C.

Figure 17 shows a Western Blot analysis of the BlaP and BlaP-HA β -lactamases using monoclonal anti-HA antibody conjugated with peroxidase. Immunorecognised proteins were visualised by enhanced chemiluminescence detection.

Figure 18 shows the titration curve of immobilised rat IgG by BlaP-HA hybrid protein. The absorbance is plotted against the quantity of IgG1 of rat anti-HA in ng.

Figure 19 shows the agarose gel where PCR amplification products of TEM-1 (197/Smal) and some hybrid TEM-1 hPLA₂ protein were loaded.

Figure 20 shows the primary structure of the hPLA₂ on which the various fragments internalised in TEM-1 are underlined (1,2 and 3).

Figure 21 shows the Potentiometric measurement of a platinum electrode where rabbit antibodies were immobilised on functionalised aniline by succinimidyl group Curve A: base line Pt/Pani/Pani-R/IgG/TemPA without substrate of the β -lactamase. Curve B: The release of protons starts with the addition of the substrate (benzylpénicilline) and the electrode potential increases proportionally with the quantity of substrate. Point 1, $2,6 \cdot 10^{-4}$ M; point 2, $2,6 \cdot 10^{-3}$ M; point 3, $2,6 \cdot 10^{-2}$ M; point 4, $2,6 \cdot 10^{-1}$ M; point 5, $5,2 \cdot 10^{-1}$ M.

[0042] The present invention will be explained in more detail in the following examples, which however do not limit the scope of protection in any way.

EXAMPLES

Methodology for constructing hybrid proteins

[0043] **Example 1: Inserting unique restriction sites in the genes of the TEM-1, BlaP and BlaL β -lactamases so as to internalise exogenous nucleotide sequences.**

[0044] The coding sequence of the β -lactamase TEM-1 which was used for internalising exogenous sequences of peptide initially contains a *KpnI* restriction site. In order to broaden the range of DNA fragments to be internalised in the carrier protein, two types of restriction cassettes were inserted into the *KpnI* site (figure 3). Among the new restriction sites, the *SmaI* site produces blunt ends compatible with all nucleotide fragments also having blunt ends. This change provides a means of making out the internalisation of the random nucleotide fragments originating with a gene of interest or the genomic DNA of any organism. *E. coli* production assays have shown that the new TEM-1 hybrid proteins retain their β -lactamase activity after the restriction cassettes have been internalised.

[0045] As figure 4 shows, the 3D structure of the BlaP β -lactamase of *B. licheniformis* is very close to that of TEM-1. This specific feature is shared by all β -lactamases of the same class. Owing to their insensitivity to the proteases action, a *SmaI* restriction site was inserted into the BlaP and BlaL coding sequences to allow exogenous nucleotide sequences to be internalised (figure 5). The restriction site was inserted in a region that was the same as the one used to internalise exogenous peptides in TEM-1 (figure 5). By the end of this process, it was possible to show that BlaP and BlaL β -lactamases retained their enzymatic activity.

[0046] **Example 2: Synthesis of hybrid proteins TEM-1 and the thermostable enterotoxin STa of Escherichia coli (TEM-STA)**

[0047] STa is a thermostable toxin mainly produced by enterotoxic *Escherichia coli* (*E. coli*) strains found either in animal as bovine or in human. STa induces severe and lethal diarrhoea in human and new borne calf respectively (Mainil J,2000). The

thermostable enterotoxin STa is a polypeptide of 18 amino acid residues. It exhibits a discrete three-dimensional structure, which is stabilised by the presence of three disulfide bonds (Gariepy et al, 1986; Shimoniski et al, 1987). The native fold of the polypeptide only mediates the toxicity.

[0048] The small size of STa does not allow its recognition by the host immune system and no anti-STa antibodies can be produced. Therefore, no protection of the host and no vertical protection (mother-foetus) are possible.

[0049] In order to develop an immune response against STa, the 18 residues can be linked to a large protein (called the carrier protein). The aim is to design a set of hybrid protein in which the STa peptide will be inserted into the TEM-1 class A β -lactamase scaffold. The different hybrid proteins will be produced in *E. coli* and purified to homogeneity. The different proteins will be injected in mice and the immune response toward the carrier protein and the insert will be studied.

[0050] **Results**

[0051] **Example 3: Selection of permissive sites.**

[0052] When performing the present invention the STa amino acid sequence was introduced into eight different positions of TEM-1. Namely, STa was placed after amino acid residues 37, 197, 198, 206, 216, 218, 232 and 260 of TEM-1 respectively (figure 6).

[0053] Those hybrid proteins were obtained by introducing the DNA coding sequence for the 18 amino acid peptide STa into the gene ampR of pBr322. The different hybrid proteins were produced in *E. coli*. The production of a stable and active enzyme was tested as follow:

[0054] a) Western blot with anti-TEM antibodies were performed to verify the production of the hybrid.

[0055] b) Determination of the MIC (minimal inhibitory concentration) of ampicillin for the different E. coli strains.

[0056] These data indicated that three different types of insertion sites could be defined. The sites in position 197, 198, 216 and 218 are permissive (detection of TEM by western blot and high MIC values). The sites in position 37, 206 and 260 are semi permissive (detection of TEM by western blot and low MIC values). The site in position 232 is a non-permissive site (no TEM-1 production and low MIC). Two positions were selected: The first one is the position 197, which is located on solvent exposed loop, and position 216 located on a buried loop.

[0057] **Example 4: Production of the different hybrid proteins.**

[0058] The hybrid proteins in which the STa sequence was inserted in position 197 and 216 were produced in E. coli. Their corresponding genes were inserted in a pTAC11 vector. The hybrids TEM197STa and TEM216STa were produced at 18°C in LG media. The enzymes were purified in three purification steps (one QFF sephaorse pH 7.5, a QFF sepharose pH 6.5 and a superdex 75 molecular sieve). The purification yield was estimated at 2 mg/liter of culture for the two enzymes.

[0059] The TEM197H (TEM-1 + amino acids inserted in position 197) was also produced in E. coli. The production was performed in a SB media. The culture was incubated at 18°C for 28 h. The enzyme was purified as described above. The purification yield was 12.6 mg/liter of culture.

[0060] Finally, as protein control, the STa sequence was introduced at the C-terminal of the glutathion-S-transferase (GST). The fusion protein was purified by affinity chromatography. The purification yield was 30 mg/liter of culture.

[0061] **Example 5: Biological activity of TEM197 STa and TEM216STa.**

[0062] 1) **Beta-lactamase activity.** Table 1 shows the steady state kinetic parameters for the different hybrid proteins and the wild type enzyme. The data indicated that the catalytic efficiencies of the different hybrids are lower than the WT.

Nevertheless, it could be demonstrated that the insertion of the STa moiety does not drastically impaired the catalytic efficiency of the TEM-1 enzyme. The conclusion is that the fold of the TEM-1 is not strongly affected by the presence of STa.

[0063] **2) Toxicity of the hybrid proteins.** The toxicity of the hybrid proteins were tested by suckling mouse assay (Gianella et al, 1976). The toxicity of STa is due to the secretion of physiological fluid in the bladder. The mass of fluid can be estimated by the determination of the ratio between the weight of the bladder and the weight of the mouse carcasse (I/C). If $I/C < 0.075$, no toxic effect is detected. If $0.075 < I/C < 0.083$ represent an intermediary effect of the toxin while an $I/C > 0.083$ indicated an strong toxic effect. Three control reactions were made by using a purified STa peptide, a supernatant of E. coli which produce (B44) or not the enterotoxin STa. The data presented in table 2 indicated that the TEM197STa and TEM216STa yielded a toxic activity.

[0064] **3) Titration of the STa toxicity.** The suckling mouse assay was performed for different protein concentrations of 197STa, 216STa and GST-STa. The I/C values were determined in function of the hybrid protein concentration (figure 7). These data indicated that the toxicity of the TEM197STa and TEM216STa were 200 fold lower than the native STa. Interestingly, the toxicity of the GST-STa was 2000 fold lower compared to STa.

[0065] **Example 6: Immunization assays.**

[0066] Six groups of three-month-old BALB/c (H-2d) female mice (Dr. Collard, Department of Animal immunology, Centre d'Economie Rurale) were used for immunisation with the different purified recombinant proteins. The mice were immunised with 50 µg of protein diluted in 50 mM sodium phosphate pH 7.2, 0.1 M NaCl (PBS buffer) containing the QuilA adjuvant (Spikoside, Isotech AB, Luleå, Sweden). Three, six weeks and 16 weeks later (day 21,42 and 112 respectively), the mice were boosted with 50 µg of the same recombinant protein. At two weeks time intervals after the first injection (day 14) and after each boosts (day 35, 56 and 127), sera were collected, pooled per group of mice, and then tested for the presence of IgG anti-TEM and anti-STa antibodies by ELISA (figure 8 A and B). The presence of IgG

anti-TEM was found in the serum collected at day 14 for TEM197STA and TEM216STA and at day 35 in the case of TEM197 H respectively. In addition, the IgG anti-Sta were produced against TEM197STA, TEM216STA and GST-STa. The immune response was detected in all the case after the second boost. The production of antibodies was always higher when the GST-STa was injected into the mice.

[0067] **Example 7: Titration curve of anti-Tem IgG antibodies and isotyping of the immune response.**

[0068] Figure 9 shows that, after the second boost, the level of anti-TEM antibodies in the different serum (with the exception of GST-STa) was equivalent. The titre was estimated to be 10000.

[0069] The nature of the different antibodies produced against TEM-1 was characterized (figure 10). The nature of the antibodies (IgG1, IgG2, IgG2a, IgG2b, IgA and IgM) was determined by ELISA. The TEM-H was used for this experiment and the day 127 - serum of mice immunised with TEM-H was chosen. The data indicated clearly that the immune response yielded a strong production of IgG1 and IgG2 antibodies. The IgA and IgM antibodies were poorly expressed. In addition the IgG2 response was further characterised. Furthermore, also the IgG2a and IgG2b were found. These data indicated that the presence of TEM-1 can induced both the Th1 and Th2 immune response.

[0070] **Example 8: Stability of the immune response versus time.**

[0071] The level of total anti-TEM IgG was measured by ELISA in serums collected at day 127 and 356 after the first injection. No boosts were realised after day 127. The results (figure 11) showed that the IgG level was always slightly higher in the serum day356 compared to those collected at day 127. Consequently, the immune response is considered to be stable for at least one year after a contact between the mice and the TEM β -lactamase.

[0072] **Example 9: Neutralization of the native STa toxicity.**

[0073] The native STa enterotoxin was incubated in presence of diluted (4 to 32 fold) serum containing the antibodies raised against GST-STa, TEM197STa and TEM260STa. The solutions were incubated for 16 hours at 4°C. The residual toxicity of the sample was estimated by "suckling mouse assay" (table 3).

[0074] The data indicated that the incubation of STa with the TEM197STa or GST-STa serum allowed a neutralisation of the biological activity of STa. Unfortunately, no clear data could be obtained for the TEM232STa.

Table 1. Kinetic parameters of the TEM197STa, TEM216STa, TEM197H and TEM-1

Antibiotics	proteins	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($\mu M^{-1}s^{-1}$)
Benzylpenicillin	TEM-1	1500	18	80
	197H	600	65	9
	197STa	56	DELAY	DELAY
	216STa	70	DELAY	DELAY
Nitrocefén	WT	930	52	18
	197H	770	170	4.5
	197STa	>560	>280	2
	216STa	N.D.	N.D.	N.D.
Cephaloridine	WT	1500	670	2.2
	197H	>1000	>1000	1
	197STa	256	172	1.5
	216STa	4.5	720	0.0062

Table 2. Determination of the toxicity of the different hybrid proteins.
 Positive control: *E. coli* B44 producing STa
 Negative control: *E. coli* non-producer of STa

Proteins	(I/C)	Toxicity
197H	0.0545 ± 0.001	-
197STa	0.107 ± 0.009	+
216STa	0.106 ± 0.011	+
GST-STa	0.074 ± 0.006	Int
<i>E. coli</i> B44	0.0925 ± 0.001	+
<i>E. coli</i>	0.0525 ± 0.001	-

Table 3. Neutralisation of the STa toxicity by the mice serum containing anti-GST-STa, TEM197STa, TEM260STa immuno-globulins. The toxicity of the serum TEM197STa, TEM197H and the serum collected at day 1 was tested by suckling mouse assay. The I/c ratio was measured with the serum diluted 8 fold. The toxicity of the protein samples TEM197STa and STa was also determined. The toxicity was measured by the I/C ratio. The STa peptide is diluted 16 fold in PBS buffer. The different serum were diluted 4 fold (4x), 8 fold (8x), 16 fold (16x) and 32 fold (32x) respectively. A positive and a negative sign indicate that the solution yielded or not a toxic response respectively.

	Samples	STa	I/C	Toxicity
Controls	197Sta	/	0.102	+
	/	STa	0.093	+
	Serum 197STa 8x	/	0.059	-
	Serum 197H 8x	STa	0.073	-
	Serum day1	STa	0.051	-
<hr/>				
Serums				
4x GST-STa	4x GST-STa	STa	0.060	-
	8x	STa	0.075	-
	16x	STa	0.091	+
	32x	STa	0.084	+
Serums	4x 197STa	STa	0.063	-
	8x	STa	0.082	+/-
	16x	STa	0.088	+
	32x	STa	0.094	+
	4x 216STa	STa	0.120- 0.054	+ -
	16x	STa	0.144- 0.074	+ -
	32x	STa 16x	0.101- 0.120	+ +

[0075] **Conclusions:** The results indicates that:

- 1) The TEM-1 β -lactamase allowed the insertion of large peptide sequences between the helices α_8 and α_9 (position 197) and also between the helices α_9 and α_{10} (site 216) without any major loss of activity and stability.
- 2) Different hybrid proteins (TEM197STa, TEM198STa, TEM216STa, TEM260STa, GST-STa and TEM197H respectively) were produced and purified to homogeneity.
- 3) The hybrid proteins TEM197STa and TEM216STa possess a rather high residual catalytic efficiency ($k_{cat}/K_M > 2 \mu M^{-1} s^{-1}$ for nitrocefin).
- 4) The different hybrid proteins exhibited a reduced toxicity compared to the native STa polypeptide.
- 5) Both Th1 and Th2 response toward the TEM enzyme but also against STa could be detected.
- 6) The serum raised against TEM197STa contains antibodies that allow a neutralisation of the biological activity of the native STa enterotoxin.

[0076] **Example 10: Construction of hybrid proteins of the TEM-1 β -lactamase where 1 to 3 repeated domains of the *Staphylococcus aureus* protein A are internalised (TEM-PA)**

[0077] The aim was to internalise one or more repeated domains of protein A in position 197 of the TEM-1 β -lactamase (197/SmaI). Protein A is composed of 5 repeated domains (fig. 12A). Each of the repeated domains of protein A is organised into three α helices (fig. 12B) interacting with the CH2 and CH3 domains of the Fc part of the IgG. This link is primarily stabilised via hydrophobic interactions. Figure 12 A, B, C.

[0078] In order to amplify the repeated domains of protein A, two primers (5'-TCAGTTAACAAATTCAACAAAGAACAAACAAAATGCT-3', SEQ ID NO: 7; 5'-TCGAAATTTTGTCTTCCTCTTTGG-3', SEQ ID NO: 8) were created which hybridise at the start and end of the gene encoding the protein A's domain B. The high similarities between the nucleotide sequences of the 5 repeated domains of

protein A allow the 5 domains to be amplified on the basis of the same set of primers. In the case of the more or less long polymerisation times, several repeated domains may be amplified via the same PCR fragment (fig. 12C). The latter feature allows a bank of nucleotidic sequences to be obtained where the five repeated domains of protein A are coded separately or in association with each others. In order to provide a highest degree of freedom for the correct folding of the domain internalised in the β -lactamase and in a way that reduces the effect of its steric constraints on the carrier protein (TEM-1), two amino acid residues were added on either side of the fragment. Ser Val for the N-terminal part and Phe Arg for the C-terminal end. It is important to stress that these primers are designed so as to amplify a fragment that preserved the reading frame of TEM-1 and of the repeated domains of protein A during the internalisation reaction.

[0079] The structural gene of the protein A, originates from a *Staphylococcus aureus* strain isolated at the Centre for Protein Engineering, was amplified by PCR with the following primers: 5'-CATATGAAAAAGAAAAACATTATTCAATTCTG-3' SEQ ID NO: 9; 5'- GGATCCTTATAGTTCGCGACGACGTCCAGCTAA-3' SEQ ID NO: 10; and in the following conditions: 95°C-180 sec, 95°C-30 sec, 55°C-60 sec, 72°C-120 sec 30 cycles, mixture of Taq polymerase/Pfu polymerase and cloned into the pGEM-T-easy plasmid.

[0080] The repeated domains of protein A (180-pb) were amplified by PCR (95°C-180 sec, 95°C-30 sec, 60°C-60 sec, 72°C-60 sec 35 cycles; mixture of Taq polymerase/Pfu polymerase) on the entire gene. An analysis of the amplification product for the repeated domains of protein A shows a ladder profile where the size of the amplified fragments is a multiple of 180-pb (fig. 12C). The PCR product was purified from an agarose gel, blunt ended by the action of the Pfu polymerase then dephosphorylated by Calf intestine phosphatase. The library of protein A PCR fragments was shotgun cloned in the TEM-1 β -lactamase gene that was cloned beforehand in the expression construct pROGENO-1 and digested by *Sma*I. The pROGENO-1 plasmid allows for a high constitutive expression of the recombinant β -lactamases in *E. coli*. In this plasmid, a unique restriction site recognised by the *Sma*I enzyme is present in position 197 of the TEM-1 β -lactamase. After transformation, the bacteria were selected via LB agar plate + Spectinomycin (100

µg/ml final) and cephaloridin (50 µg/ml). The cephaloridin is an antibiotic with a β-lactam ring hydrolysed by the TEM-1 wild-type and recombinant protein.

[0081] After plate selection, a colony PCR reaction analysis was performed to control the size of the TEM-1 gene. Towards this end, primers outcrossing upstream and downstream from the coding sequence of the mature form of TEM-1 (5'-cgggagctcaggctacccagaaacgctggtg-3'; 5'-cgggattctcaccaatgcttaatcagtgaggcacc (SEQ ID NO 11 and SEQ ID NO:12); 95°C-180 sec; 95°C-30 sec, 65°C-60 sec, 72°C-90 sec 35 cycles, mixture of Taq polymerase/pfu polymerase) were used. In a population of 30 clones, all of them were bigger than the encoding TEM-1 gene. On the agarose gel shown in figure 12D, the PCR fragments coding for the TEM-PA hybrid proteins have been loaded, where 1 to 3 repeated domains of the protein A were internalised.

[0082] The productions of the various TEM-PA hybrid proteins were achieved in the *E. coli* JM109 strain. After a 24-hours fermentation at 37°C in a rich medium, TEM-PA hybrid proteins were overproduced in the periplasm of the bacteria. However the SDS-PAGE gel analysis shows that the hybrid proteins are partly proteolysed during their biosynthesis. The hybrid proteins were then affinity chromatography purified on IgG-sepharose (fig. 12E) until homogeneity was reached. This showed that the domains of the protein A internalised in TEM-1 retained their affinity for the antibodies Fc region. Hydrolysis tests on the chromogenic substrate nitrocefin (red cephalosporin, antibiotic with a β-lactam ring) reveal that the hybrid proteins also retain β-lactamase activity after purification.

[0083] In order to check if the TEM-PA chimeras can be used to quantify the antibodies, ELISAs were developed in which increasing levels of rabbit IgG were immobilised on a polystyrene microplate by alkaline pH absorption (Na_2CO_3 1.59 g/l, NaHCO_3 2.93 g/l, pH 9.6). After saturation (PO_4^- 50 mM, NaCl 150 mM, Tween-20 0.05%, Non-fat dried Milk 3%, pH 7.5), a fixed amount of TEM-PA hybrid protein was added (PO_4^- 50 mM, NaCl 150 mM, Tween-20 0.05%, Non-fat dried Milk 1%, pH 7.5) where one repeated domain of the protein A was internalised. After washing (3X - PO_4^- 50 mM, NaCl 150 mM, Tween-20 0.05%, Non-fat dried Milk 1%, pH 7.5; 1x - PO_4^- 50 mM, NaCl 150 mM, pH 7.5), red cephalosporin (100 µM) was added. In this test, the β-lactamase activity gave rise to a red colour which was followed at 482 nm.

Figure 13 shows the possibility of detecting between 10 and 100 ng of rabbit IgG after a 1h development. The sensitivity of this test should be further increased by using a chimera protein containing several repeated domains of the protein A.

[0084] **Example 11 Construction of the hybrid proteins of the BlaP β -lactamase where 1 to 3 repeated domains of *Staphylococcus aureus* protein A are internalised (BlaP-PA).**

[0085] The BlaP-PA chimerical partner were constructed and purified according to the same procedure described in example 10 for the TEM-1 β -lactamase. The BlaP β -lactamase is used as carrier of peptide fragment and the exogenous peptides are internalised at the site 211 (211/Smal). The resulting hybrid β -lactamase retains its activity and also the internalised protein A is functional.

[0086] **Example 12 Construction of the hybrid proteins of the TEM-1 β -lactamase where the B1 and/or B2 domain or domains of the *Streptococcus pyogenes* protein G are internalised (TEM-PG).**

[0087] The aim was to internalise one or more repeated domains of the protein G in position 197 of the TEM-1 β -lactamase (197/Smal). The protein G is composed of 2 repeated domains, called B1 and B2. They confer an affinity for the antibodies Fc region (fig. 14A). Each of the two domains is organised with a β sheet and a α helices (fig. 14B) interacting with the CH2 and CH3 domains of the Fc region of the IgG.

[0088] The methodology used to construct TEM-PG hybrid proteins is exactly the same as the one described in example 10 for TEM-PA, apart from the following observations:

[0089] The primers used are:

5'-GGCTGTACTTACAAATTAATCCTTAATGGTAAAACATTG-3' (SEQ ID NO: 13)
and 5'-CTCTCTTCAGTTACCGTAAAGGTCTTAGTCGC-3' (SEQ ID NO: 14). The structural gene used as a matrix during the PCR originates from the genomic DNA of *Streptococcus pyogenes* strain isolated at the Centre for Protein Engineering. In order to reduce the steric constraints the following amino acid residues were added

on either side of the fragment. Gly Cys for the N-terminal part and Arg Glu for the C-terminal end.

[0090] At the end of the screening stages, TEM-1 β -lactamases was isolated where 1 or 2 repeated domains of the protein G were internalised (fig. 14C). The affinity of the TEM-PG chimera proteins for the IgG immobilised on the sepharose column shows that the internalised domains of proteins G are always functional (fig. 14E). Hydrolysis tests on the chromogenic substrate nitrocefin (antibiotic with a β -lactam nucleus) show that the TEM-PG chimeras purified on IgG-sepharose retain the β -lactamase activity. The tests showed that the internalised domains of protein G were functional.

[0091] **Example 13 Construction of the hybrid proteins of the BlaP β -lactamase where the B1 and/or B2 domain or domains of the Streptococcus pyogenes protein G are internalised (BlaP-PG).**

[0092] The BlaP-PG chimera proteins were constructed and purified according to the same procedure as the one described in example 11 and 12. The BlaP β -lactamase (211/Smal) was used as a carrier protein. The chimeras purified on IgG-sepharose retain the β -lactamase activity. The tests also showed that the internalised domains of protein G were functional.

[0093] **Example 14 Construction of the hybrid proteins of the BlaP β -lactamase where a linear epitope of the Influenza virus hemagglutinin is internalised (BlaP-HA)**

[0094] In order to create this hybrid protein, complementary primers (5'-AGGTTTATCCATACGACGTCCGGACTACGCCACAAT-3' SEQ ID NO: 15, 5'-AGTTGTGGCGTAGTCGGGACGTCGTATGGATAAACCT-3' SEQ ID NO: 16) were created that code for a linear epitope (HA) of the Influenza virus hemagglutinin (YPYDVPDYA). Here and there on the coding region for the epitope, two codons which code for Arg Phe and Thr Thr amino acids were added at the beginning and at the end of epitope, respectively. In this experiment, the polypeptide internalised in BlaP comprises 15 amino acids 4 of which are used for steric constraints (fig. 15).

[0095] The two primers were hybridised and inserted into the BlaP gene (211/Smal) which was cloned beforehand in the pROGENO-1 expression vector and digested by *Smal*. After transformation, the bacteria were selected on LB agar plate + Spectinomycin (100 µg/ml final) and cephaloridin (50 µg/ml). At the end of the screening stage, BlaP β-lactamases were isolated where the epitope HA was internalised. The BlaP-HA hybrid protein was then overproduced in *E. coli* as a result of the pROGENO-1 expression vector. After extracting the periplasmic fraction by cold osmotic shock, the BlaP-HA chimera was partly purified on S-sepharose Fast Flow (SFF) in sodium acetate buffer (20 mM, pH 4.5) and eluted with a NaCl gradient. The SDS-PAGE gel featured in figure 16 shows the BlaP and BlaP-HA hybrid protein after SFF purification.

[0096] The antigenicity of the HA epitope internalised in BlaP was controlled by a Western Blot reaction using a specific monoclonal antibody of this hemagglutinin's epitope (rat anti-HA IgG1, 3F10, Roche). Figure 17 shows that the monoclonal anti-HA antibody recognised the denatured BlaP-HA chimera and no cross-reaction with wild-type BlaP was detected.

[0097] The aforementioned Western Blot experiment shows that the HA epitope is recognised when the BlaP-HA chimera protein is denatured. In order to check that the anti-HA antibody also recognises the HA epitope when it is internalised in a non-denatured BlaP form an ELISA reaction was performed in which increasing quantities of rat anti-HA IgG1 were immobilised (not linked to the peroxidase, 3C10, Roche) (Na_2CO_3 1.59 g/l, NaHCO_3 2.93 g/l, pH 9.6). After saturation (Tris 50 mM, NaCl 0.5 M, Tween-20 0.05%, Non-fat dried Milk 1%, pH 7.6), a fixed amount of BlaP-HA chimera protein was added (Tris 50 mM, NaCl 150 mM, Tween-20 0.05%, Non-fat dried Milk 1%, pH 7.6). After washing (3x - Tris 50 mM, NaCl 0.5 M, Tween-20 0.05%, Non-fat dried Milk 1%, pH 7.6; 1x - Tris 50 mM, NaCl 0.5 M, pH 7.6), red cephalosporin (100 µM) was added. Figure 18 shows the possibility of detecting between 10 and 100 ng of rat anti-HA IgG1 after 1h of development. This demonstrates that β-lactamase activity can be used to quantify interaction between antibody and antigen/epitope fragment.

[0098] Example 15 Construction of the hybrid proteins of the TEM-1 β -lactamase where fragments of human phospholipase - type II, hPLA₂ are internalised (TEM-PLA2)

[0099] In order to internalise random fragments of the human phospholipase - type II gene, hPLA₂ in the TEM-1 β -lactamase, the hPLA₂ gene was amplified by PCR. The PCR reaction was applied with the following primer: 5'-CTCGAGAAAAGAAATTGGTGAATTCCAC-3' (SEQ ID NO: 17) and 5'-GCAACGTGGAGTGCTCCCTCTGCAGTGT-3' (SEQ ID NO: 18) (95°C-180 sec; 95°C-30 sec, 65°C-60 sec, 72°C-60 sec 35 cycles, mixture of Taq polymerase/Pfu polymerase). After purification, the PCR product was digested with DNase so as to produce DNA fragments between 50 and 430 bp. This stage may be replaced by a nebulisation reaction. The DNA fragments were then purified, blund ended by the action of the Pfu polymerase then dephosphorylated by Calf intestine phosphatase and shotgun cloned in the TEM-1 β -lactamase carried by the expression vector pROGENO-1. At the end of the screening stages, several TEM-1 β -lactamases chimeras were isolated where fragments of varying sizes originated from the hPLA₂ gene were internalised (figure 19). In the case of three of them, sequencing reaction was applied in order to identify the hPLA₂ regions which were internalised (figure 20). In the case of the first chimera, the N-terminal domain of the hPLA₂ (residues 1 to 45) had been internalised. This peptide fragment contains the calcium binding site and some residues of the active site. In the second chimera, an internal fragment of the hPLA₂ (residues 40 to 66), covering the residues of the active site were internalised. In the third chimera, all the hPLA₂ protein, except for the 20 last residues, were internalised (figure 20). It is important to stress that the β -lactamase activity is retained for each one of the chimera selected. Trials involving the binding of calcium and measuring phospholipase activity were successful.

[00100] Example 16 Construction of a hybrid protein of the BlaP β -lactamase where fragments of multimerised polypeptides comprising three amino acids repeated in tandem and presenting an affinity for bacterial endotoxins are internalised (BlaP-LPS)

[00101] In order to construct a new LPS-binding peptide, first of all two complementary primers corresponding to the LPS-binding amino acid sequence (Pro

Ile Ile Lys Leu Leu Lys Leu Leu Lys Leu Leu Arg Arg Lys Leu Leu Lys Leu Leu Pro Asp Gln Glu Phe Lys Gln) were hybridised. Primer sequence: 5'-CCGATCATCAAACCTCTCAAGCTGCTTAAACTCCTGCGCCGGAAACTTCTCAAG CTGCTTAAACTCCTGCCGGATCAGGAGTTAACGCAG-3' and 5'-CTGCTTAAACTCCTGATCCGGCAGGAGTTAACGCAGCTTGAGAAGTTCCGGCG CAGGAGTTAACGCAGCTTGAGAAGTTGATGATCGG-3'. Hybridisation is achieved by heat denaturation followed by a slow cooling stage. Double stranded oligonucleotide was inserted in the gene of the BlaP β -lactamase that was cloned beforehand in the expression vector pROGENO-1 and digested by SmaI. After transformation, the bacteria were selected on LB agar plate + Spectinomycin (100 μ g/ml final) and cephaloridin (50 μ g/ml). At the end of the screening stages, BlaP β -lactamases were isolated where LPS-binding domain was internalised. The affinity of the BlaP-LPS chimera proteins for LPS is now being characterised. Hydrolysis tests on the chromogenic substrate nitrocefin reveal that the BlaP-LPS chimeras also retain β -lactamase activity.

[00102] Example 17 Exploitation of the hybrid protein TEM-PA in electrobiochemical biosensor system

[00103] The term biosensor has been applied to devices either used to monitor living systems, or to incorporate biologic or biomimetic elements. Here, in this application a "biosensor" is used in the context of a sensor incorporating a biological element such as an enzyme, antibody, nucleic acid, microorganism or cell.

[00104] The usual aim of a biosensor is to produce either discrete or continuous digital electronic signals which are proportional to a single analyte or a related group of analytes.

[00105] Experimental procedure: A polyaniline (Pani) film is electropolymerised on a platinum foil (1 x 0.5 cm) on the basis of a 1 M HClO₄ solution containing 0.1 M aniline, by potential sweeps between -0.2 and 0.8 V/SCE to 20 mV/s. The Pani film is functionalised in an electrochemical bath containing a 1 M HClO₄ solution, 0.05M in 3-aminophenol and 0.05 M aniline with potential sweeps between -0.2 and 0.8 V/SCE to 20 mV/s. The film is then immersed in an acetonitrile solution (4 ml) containing 0.2

ml of triethylamine, 0.04 g of disuccinimidyl carbonate and 0.01g of dimethylaminopyridine (DMAP) for one night at ambient temperature.

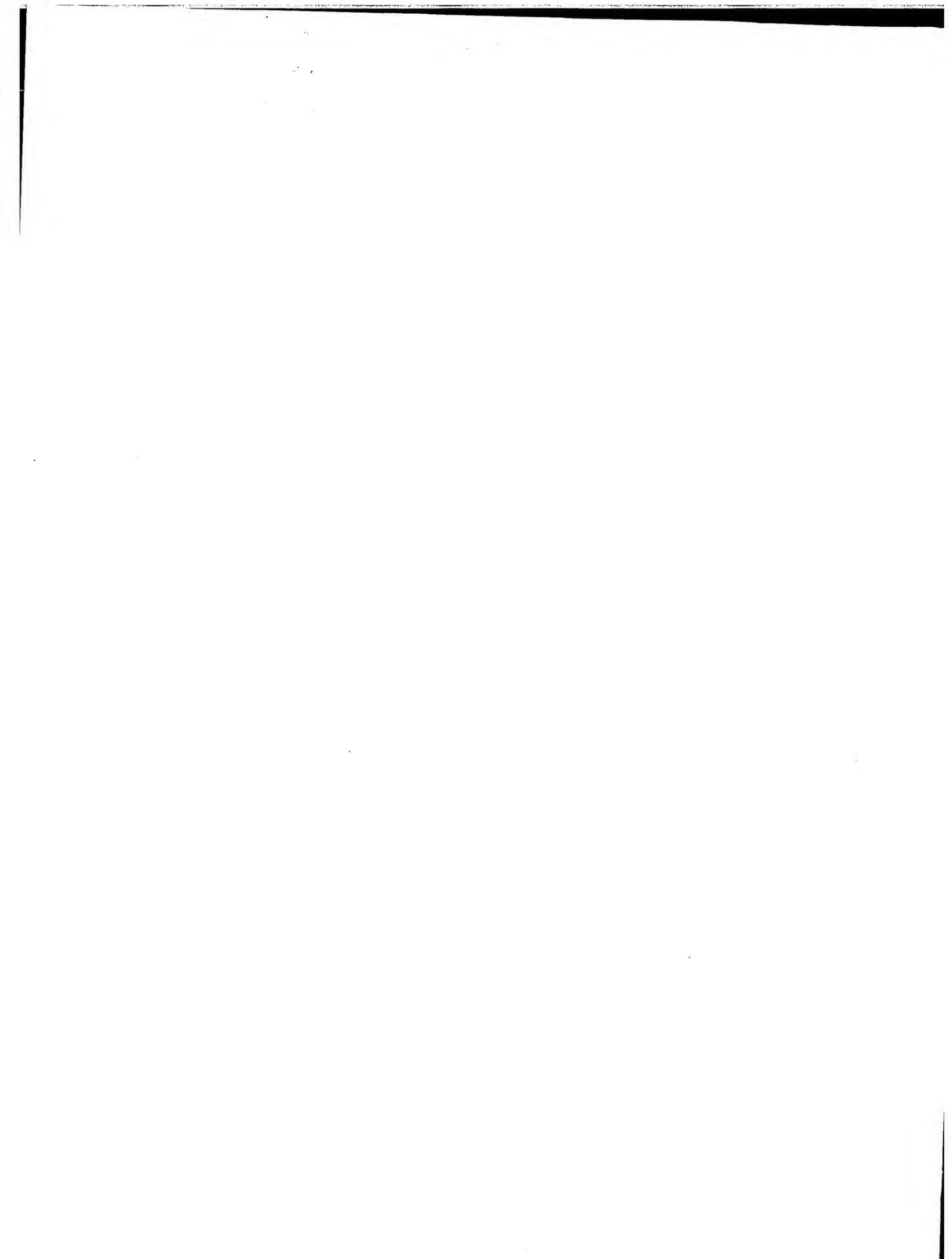
[00106] The rabbit antibodies (IgG) are immobilised on the functionalised film for one night in a pH=8 phosphate buffer and 300 µl of a 4mg/ml IgG solution. The IgG assay is achieved as follows: 50 µl of a solution of the TEM-PA hybrid protein (1µg/µl) are deposited on the electrode (Pt/Pani/Pani-R/IgG) for 15 min, the electrode is rinsed by 3 x 5 ml pH=8 phosphate buffer. The potentiometric measurement is achieved in a simple compartment cell containing 4.5 ml 0.1M NaCl solution and a calomel reference electrode (SCE). The working electrode and reference are connected to a multimeter and all the potential values are collected every 30 seconds (fig. 21). Benzylpenicillin is added every minute so that each addition produces a substrate concentration in the bath ranging from $2.6 \cdot 10^{-4}$ M to $2.6 \cdot 10^{-1}$ M.

[00107] **References**

- 1: Legendre D, Vucic B, Hougardy V, Girboux AL, Henrioul C, Van Haute J, Soumillion P, Fastrez J. TEM-1 β -lactamase as a scaffold for protein recognition and assay. *Protein Sci.* 2002 Jun;11(6):1506-18.
- 2: Legendre D, Soumillion P, Fastrez J. Engineering a regulatable enzyme for homogeneous immunoassays. *Nat Biotechnol.* 1999 Jan;17(1):67-72.
- 3: Vanwetswinkel S, Touillaux R, Fastrez J, Marchand-Brynaert J. Bifunctional activity labels for selection of filamentous bacteriophages displaying enzymes. *Bioorg Med Chem.* 1995 Jul;3(7):907-15.
- 4: Vanwetswinkel S, Fastrez J, Marchand-Brynaert J. Synthesis of new sulfonylamido-penicillanic acid sulfones inhibitors of β -lactamases. *J Antibiot (Tokyo)*. 1994 Sep;47(9):1041-51.
- 5: Soumillion P, Jespers L, Bouchet M, Marchand-Brynaert J, Winter G, Fastrez J. Selection of β -lactamase on filamentous bacteriophage by catalytic activity. *J Mol Biol.* 1994 Apr 8;237(4):415-22.

- 6: Guillaume G, Vanhove M, Lamotte-Brasseur J, Ledent P, Jamin M, Joris B, Frere JM. Site-directed mutagenesis of glutamate 166 in two β -lactamases. Kinetic and molecular modeling studies. *J Biol Chem.* 1997 Feb 28;272(9):5438-44.
- 7: Galarneau A, Primeau M, Trudeau LE, Michnick SW. β -lactamase protein fragment complementation assays as *in vivo* and *in vitro* sensors of protein-protein interactions. *Nat Biotechnol.* 2002 Jun;20(6):619-22.
- 8: Hakimelahi GH, Shia KS, Pasdar M, Hakimelahi S, Khalafi-Nezhad A, Soltani MN, Mei NW, Mei HC, Saboury AA, Rezaei-Tavirani M, Moosavi-Movahedi AA. Design, synthesis, and biological evaluation of a cephalosporin-monohydroguaiaretic acid prodrug activated by a monoclonal antibody- β -lactamase conjugate. *Bioorg Med Chem.* 2002 Sep;10(9):2927-32.
- 9: Melton RG, Sherwood RF. Antibody-enzyme conjugates for cancer therapy. *J Natl Cancer Inst.* 1996 Feb 21;88(3-4):153-65. Review.
- 10: Spotts, James M.; Dolmetsch, Ricardo E.; Greenberg, Michael E. Division of Neuroscience, John F. Enders. Time-lapse imaging of a dynamic phosphorylation-dependent protein-protein interaction in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America* (2002), 99(23), 15142-15147. CODEN: PNASA6 ISSN: 0027-8424.
- 11: Simon H, Voronov AV, Kvetkauskaite R, Lang H.J. A simple ELISA procedure for HIV-1 based on the enzyme β -lactamase. *Immunol Methods* 1991 Jun 24;140(1):85-92
- 12: Geetha PB, Ghosh SN, Gupta NP, Shaikh BH, Dandawate CN. Enzyme linked immunosorbent assay (ELISA) using β -lactamase for the detection of antibodies to KFD virus. *Indian J Med Res* 1980 Mar;71:329-32
- 13: Patel SB, Khatkhatay I, Desai MP, Betrabet SS, Toddywalla VS. A sensitive ELISA for 6 β -hydroxycortisol in urine using enzyme penicillinase (β -lactamase). *J Steroid Biochem Mol Biol* 1994 Feb;48(2-3):293-

- 14: Hayes F, Hallet B, Cao Y. Insertion mutagenesis as a tool in the modification of protein function. Extended substrate specificity conferred by pentapeptide insertions in the omega-loop of TEM-1 β -lactamase. *J Biol Chem* 1997 Nov 14;272(46):28833-6.
- 15: Hallet B, Sherratt DJ, Hayes F. Pentapeptide scanning mutagenesis: random insertion of a variable five amino acid cassette in a target protein. *Nucleic Acids Res* 1997 May 1;25(9):1866-7;
- 16: Si Jae Park and San Yup Lee Efficient recovery of secretory recombinant protein from protease negative mutant *Escherichia coli* strains. *Biotechnology Techniques*, Vol. 12, N°11, November 1998, pp. 815-818
- 17: Baneyx F, Schmidt C, Georgiou G. Affinity immobilization of a genetically engineered bifunctional hybrid protein. *Enzyme Microb. Technol.* 1990. 12, 337-42.
- 18: Yuqiang Wang, Huiling Yuan, Susan C Wright , Hong Wang and James W Lerrick Synthesis and preliminary cytotoxicity study of a cephalosporin-CC-1065 analogue prodrug. *BMC Chemical Biology* 2001.



Claims

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(67)

1. A recombinant nucleotide sequence which codes upon expression for at least a part of a hybrid β -lactamase class A protein, characterized in that the β -lactamase class A protein is bearing at least one heterologous sequence in a region located between two neighboring alpha helices of the β -lactamase sequence, wherein the region is selected from:
 - a) the region forming a juncture between alpha helix 8 and alpha helix 9 of the TEM-1 β -lactamase;
 - b) the region forming a juncture between the alpha helices of homologous β -lactamases class A, said alpha helices corresponding to the alpha helix 8 and alpha helix 9 of the TEM-1 β -lactamase.
2. The recombinant nucleotide sequence according to claim 1, wherein the hybrid β -lactamase is possessing an activity selected from
 - a) hydrolysing β -lactams;
 - b) binding covalently and in a stable manner to derivatives of β -lactams and inhibitors.
3. The recombinant nucleotide sequence according to claim 1 or 2, wherein the hybrid β -lactamase retains its activity of hydrolysing β -lactams at least partially.
4. The recombinant nucleotide sequence according to any one of claims 1 to 3, wherein the three-dimensional structure of the β -lactamase portion of the hybrid β -lactamase is homologue to the three-dimensional structure of the TEM-1 β -lactamase.
5. The recombinant nucleotide sequence according to any one of claims 1 to 4, wherein the nucleotide sequence coding for the β -lactamase sequence is selected from:
 - a) nucleotide sequence coding for the β -lactamase TEM-1 (SEQ ID NO: 1)

- b) nucleotide sequence coding for the β -lactamase BlaP (SEQ ID NO: 2);
 - c) nucleotide sequence coding for the β -lactamase BlaL (SEQ ID NO: 3);
 - d) nucleotide sequences which hybridise under stringent conditions to the nucleotide sequences of any one of a), b) or c) or fragments thereof.
5. 6. The recombinant nucleotide sequence according to any one of claims 1 to 5, wherein the heterologous sequence is partially or fully replacing the region between alpha helix 8 and alpha helix 9 or the region between alpha helix 9 and alpha helix 10.
7. The recombinant nucleotide sequence according to any one of claims 1 to 6, wherein the heterologous sequence has a length of 11 or more amino acid residues, (preferably in the range of 11 to 5000 amino acid residues, more preferably in the range of 11 to 3000 amino acid residues, more preferred in the range of 11 to 2000 amino acid residues, further preferred in the range of 11 to 1000, even more preferred in the range of 11 to 300, most preferred in the range of 18 to 200 amino acid residues).
10. 8. The recombinant nucleotide sequence according to any one of claims 1 to 4, wherein the hybrid β -lactamase is a bifunctional protein.
15. 9. The recombinant nucleotide sequence according to any one of claims 1 to 8, wherein the heterologous sequence is related to a function.
20. 10. The recombinant nucleotide sequence according to any one of claims 1 to 9, wherein the function of the heterologous sequence as such is selected from: being an epitope, being a specific binding partner for antibodies, being specifically recognized and bound by antibodies, having a binding affinity to earth alkali and metal ions, having enzymatic activity, being a toxin (STa heat-stable enterotoxin of *E. coli*), bearing a glycosylation site, bearing a glycosylated peptide, being a specific binding partner for any polypeptide or any ligand, having a binding affinity to dsDNA and ssDNA or RNA (having a binding affinity to nucleotide and polynucleotide).
- 25.

11. The recombinant nucleotide sequence according to any one of claims 1 to 10, wherein the heterologous sequence is selected from the group: STa (heat stable enterotoxin of *Escherichia coli*, SEQ ID NO: 21), protein A of *Staphylococcus aureus*, (SEQ ID NO: 23 and 25), protein G of *Streptococcus pyogenes*, (SEQ ID NO: 27 and 29), a linear antigenic determinant of the hemagglutinin of the Influenca virus (SEQ ID NO: 31), a fragment of human phospholipase – type II (hPLA₂) (SEQ ID NO: 33), LPS binding amino acid sequence (SEQ ID NO: 35), and nucleotide sequences which hybridise under stringent conditions to said nucleotide sequences or fragments thereof.
12. A recombinant polypeptide which is encoded by the recombinant nucleotide sequence according to any one of claims 1 to 11.
13. A recombinant polypeptide comprising at least a part of a β-lactamase class A protein, characterized in that the β-lactamase class A protein is bearing at least one heterologous sequence in a region located between two neighboring alpha helices of the β-lactamase sequence, wherein the region is selected from:
 - a) the region forming a juncture between alpha helix 8 and alpha helix 9 of the TEM-1 β-lactamase;
 - b) the region forming a juncture between the alpha helices of homologous β-lactamases class A, said alpha helices corresponding to the alpha helix 8 and alpha helix 9 of the TEM-1 β-lactamase.
14. The recombinant polypeptide according to claim 13, wherein the hybrid β-lactamase is possessing an activity selected from
 - a) hydrolysing β-lactams;
 - b) binding covalently and in a stable manner to derivatives of β-lactams and inhibitors.

15. The recombinant polypeptide according to claim 13 or 14, wherein the hybrid β -lactamase retains its activity of hydrolysing β -lactams at least partially.
- 5 16. The recombinant polypeptide according to any one of claims 13 to 15, wherein the three-dimensional structure of the β -lactamase portion of the hybrid β -lactamase is homologue to the three-dimensional structure of the TEM-1 β -lactamase.
- 10 17. The recombinant polypeptide according to any one of claims 13 to 16, wherein the β -lactamase sequence is selected from:
 - a) β -lactamase TEM-1 (SEQ ID NO: 4)
 - b) β -lactamase BlaP (SEQ ID NO: 5);
 - c) β -lactamase BlaL (SEQ ID NO: 6).
- 15
18. The recombinant polypeptide according to any one of claims 13 to 17, wherein the heterologous sequence is partially or fully replacing the region between alpha helix 8 and alpha helix 9 or the region between alpha helix 9 and alpha helix 10.
- 20 19. The recombinant polypeptide according to any one of claims 13 to 18, wherein the heterologous sequence has a length of 11 or more amino acid residues, (preferably in the range of 11 to 5000 amino acid residues, more preferably in the range of 11 to 3000 amino acid residues, more preferred in the range of 11 to 2000 amino acid residues, further preferred in the range of 11 to 1000, even more preferred in the range of 11 to 300, most preferred in the range of 18 to 200 amino acid residues).
- 25
20. The recombinant polypeptide according to any one of claims 13 to 19, wherein the hybrid β -lactamase is a bifunctional protein.

21. The recombinant polypeptide according to any one of claims 13 to 20, wherein the heterologous sequence is related to a function.
22. The recombinant polypeptide according to any one of claims 13 to 21, wherein the function of the heterologous sequence as such is selected from: being an epitope, being a specific binding partner for antibodies, being specifically recognized and bound by antibodies, having a binding affinity to earth alkali ions and metal ions, having enzymatic activity, being a toxin (STa heat-stable enterotoxin of *E. coli*), bearing a glycosylation site, bearing a glycosylated peptide, being a specific binding partner for any polypeptide or any small ligand, having a binding affinity to dsDNA and ssDNA or RNA (having a binding affinity to nucleotide and polynucleotide).
5
10
23. The recombinant polypeptide according to any one of claims 13 to 22, wherein the heterologous sequence is selected from the group: STa (heat stable enterotoxin of *Escherichia coli*) (SEQ ID NO: 22), protein A of *Staphylococcus aureus* (SEQ ID NO: 24 and 26), protein G of *Streptococcus pyogenes* (SEQ ID NO: 28 and 30), a linear antigenic determinant of the hemagglutinin of the Influenca virus (SEQ ID NO: 32), a fragment of human phospholipase – type II (hPLA₂) (SEQ ID NO: 34), LPS binding amino acid sequence (SEQ ID NO: 36).
15
20
24. Use of the recombinant nucleotide sequence of claims 1 to 11 or the recombinant polypeptide of any one of claims 12 to 23 for vaccination.
25. Use of the recombinant nucleotide sequence of claims 1 to 11 or the recombinant polypeptide of any one of claims 12 to 23 for raising antibodies against the heterologous sequence.
25
26. Use of the recombinant nucleotide sequence of claims 1 to 11 or the recombinant polypeptide of any one of claims 12 to 23 for epitope mapping.

27. Use of the recombinant nucleotide sequence of claims 1 to 11 or the recombinant polypeptide of any one of claims 12 to 23 for affinity chromatography.
28. Use of the recombinant nucleotide sequence of claims 1 to 11 or the recombinant polypeptide of any one of claims 12 to 23 for the concentration and/or purification of antibodies directed against the heterologous sequence.
5
29. Use of the recombinant nucleotide sequence of claims 1 to 11 or the recombinant polypeptide of any one of claims 12 to 23 for the qualitative and/or quantitative detection of molecules binding to the heterologous sequence.
10
30. Use of according to claim 29, wherein the molecules binding to the heterologous sequence are antibodies or antibody fragments, polypeptides, dsDNA, ssDNA, RNA or small ligands.
15
31. Pharmaceutical compositions comprising a recombinant polypeptide of any one of claims 12 to 23.
20
32. Use of a recombinant polypeptide of any one of claims 12 to 23 for the manufacture of a medicament for the preventive and/or therapeutic treatment of diseases selected from the group cancer, viral diseases and bacterial diseases (or infection diseases), autoimmune diseases and allergy.
25
33. The use of a recombinant polypeptide of any one of claims 12 to 23 for the development of a medicament
34. A method for screening a compound for treatment, prevention and/or diagnosis of a disease which comprises the step of detecting interaction
25

between the homologous sequence of the hybrid β -lactamase according to claims 12 to 23 and a protein or polypeptide which binds to the homologous sequence in presence of a compound to be tested.

- 5 35. The method according to claim 34, wherein the compound tested is selected as a candidate of an effective medicament when the compound has an effect on the interaction between the homologous sequence and the polypeptide which binds to the homologous sequence.
- 10 36. The method according to claims 34 or 35, which comprises the steps of:
 - a) subjecting the recombinant polypeptide of any one of claims 12 to 23 and a polypeptide which binds to the homologous sequence to interaction with each other in the presence of the compound to be tested;
 - b) subjecting the recombinant polypeptide of any one of claims 12 to 23 and a polypeptide which binds to the homologous sequence to interaction with each other in the absence of the compound to be tested;
 - c) detecting the interactions in the steps a) and b), and
 - d) comparing the interactions in the steps a) and b) to chose the compound having an effect on the interaction as a candidate of an effective medicament.
- 15 37. A biologic sensor comprising a recombinant polypeptide of any one of claims 12 to 23.
- 20 38. The biologic sensor according to claim 33, wherein the biologic sensor is comprising a carrier bearing a recombinant polypeptide of any one of claims 12 to 23.
- 25



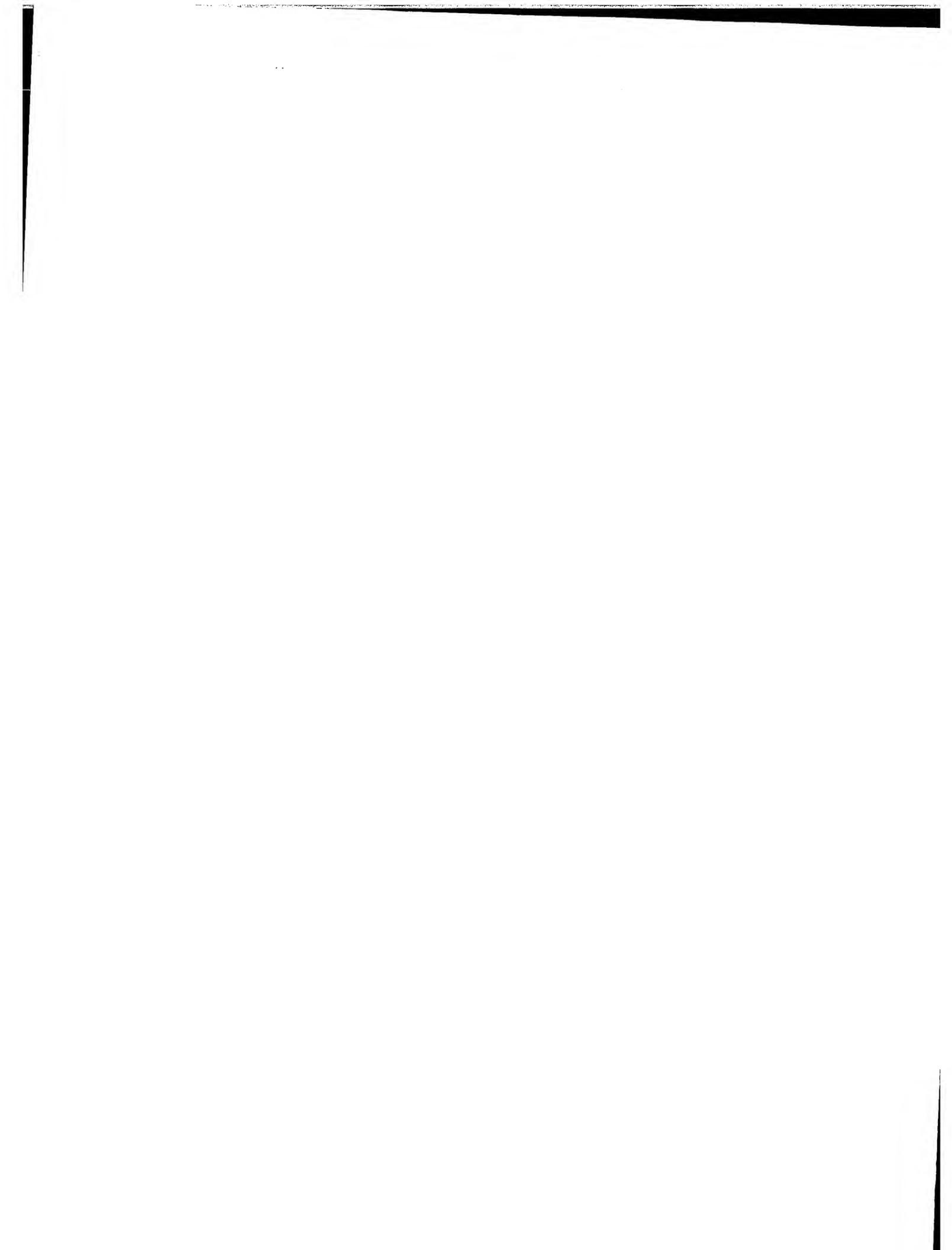
11. 02. 2004

Abstract

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The present invention refers to a recombinant nucleotide sequence which codes upon expression for at least a part of a hybrid β -lactamase class A protein, wherein in that the β -lactamase class A protein is bearing at least one heterologous sequence in a region located between two neighboring alpha helices of the β -lactamase sequence, wherein the region is selected from:

- a) the region forming a juncture between alpha helix 8 and alpha helix 9 of the TEM-1 β -lactamase;
- b) the region forming a juncture between the alpha helices of homologous β -lactamases class A, which correspond to the alpha helix 8 and alpha helix 9 of the TEM-1 β -lactamase.



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Fig. 1

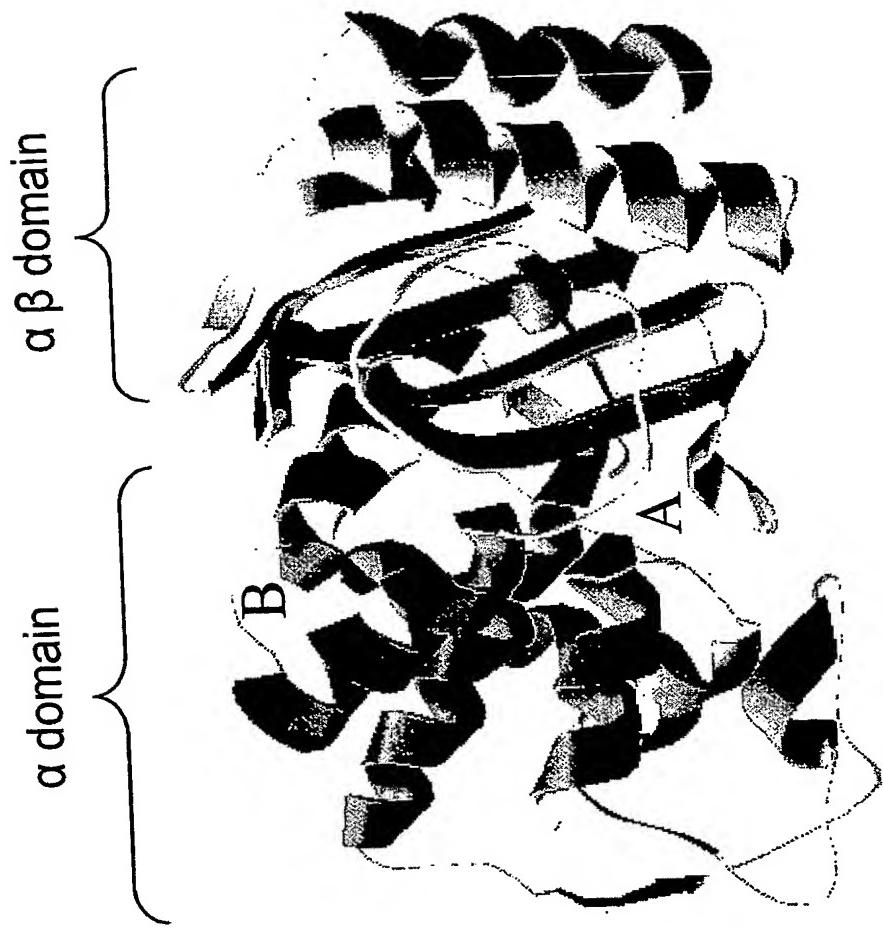


Fig. 2

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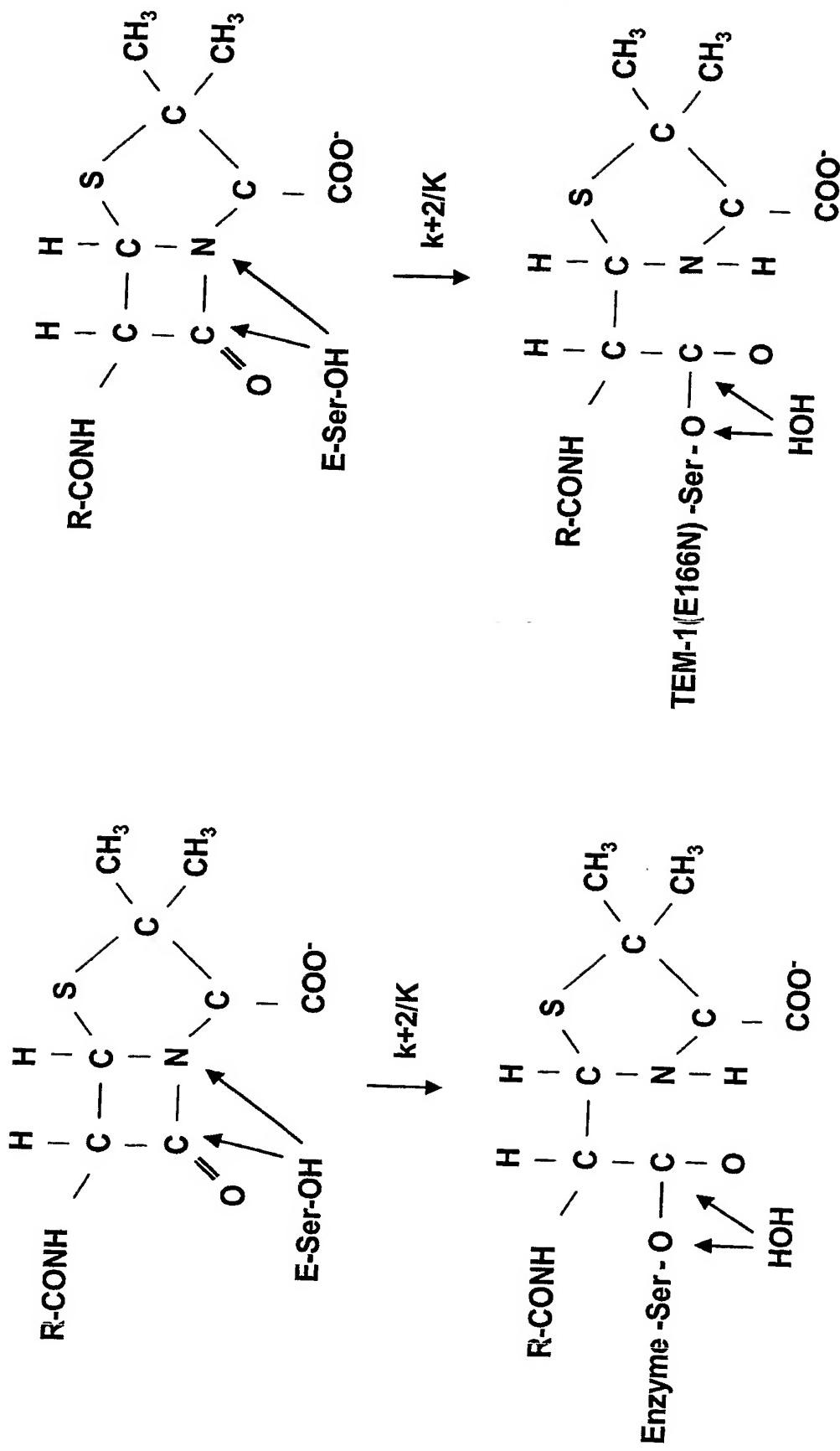
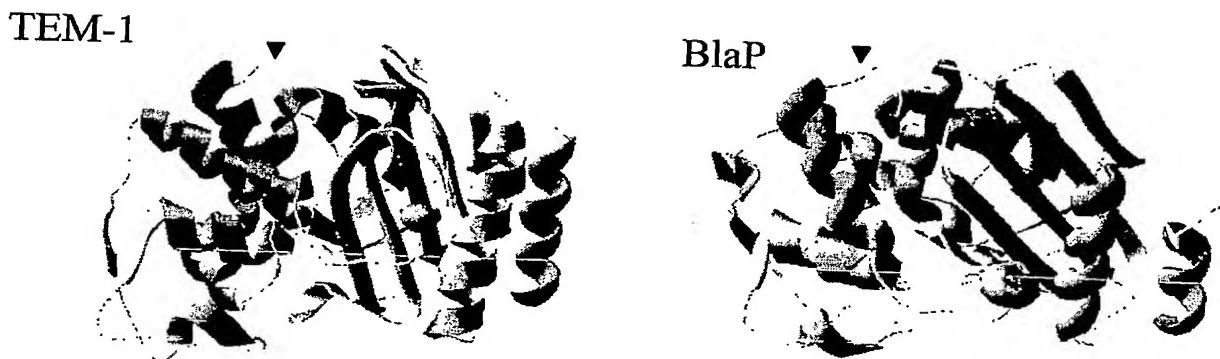


Fig. 3

Fig. 4**Fig. 5**

BlaP Wt	Ala Leu Glu Asp Lys Leu Pro Ser Glu Lys GCT - CTT - GAA - GAT - AAA - CTT - CCA - AGT - GAA - AAA
BlaP 211 SmaI	Ala Leu Glu Asp <i>Pro</i> <i>Gly</i> Lys Leu Pro Ser Glu Lys GCT - CTT - GAA - GAT - <u>CCC</u> - <u>GGG</u> - AAA - CTT - CCA - AGT - GAA - AAA <i>SmaI</i>
BlaL WT	Val Glu Asp Gly Glu Lys Ala Ala Leu Ala GTC - GAG - GAC - GGC - GAG - AAG - GCC - GCC - CTC - GCG
BlaL 203 SmaI	Val Glu Asp Gly Glu <i>Asp</i> <i>Ile</i> Lys Ala Ala Leu Ala GTC - GAG - GAC - GGC - GAG - <u>GAT</u> - <u>ATC</u> - AAG - GCC - GCC - CTC - GCG <i>EcoRV</i>

Fig. 6

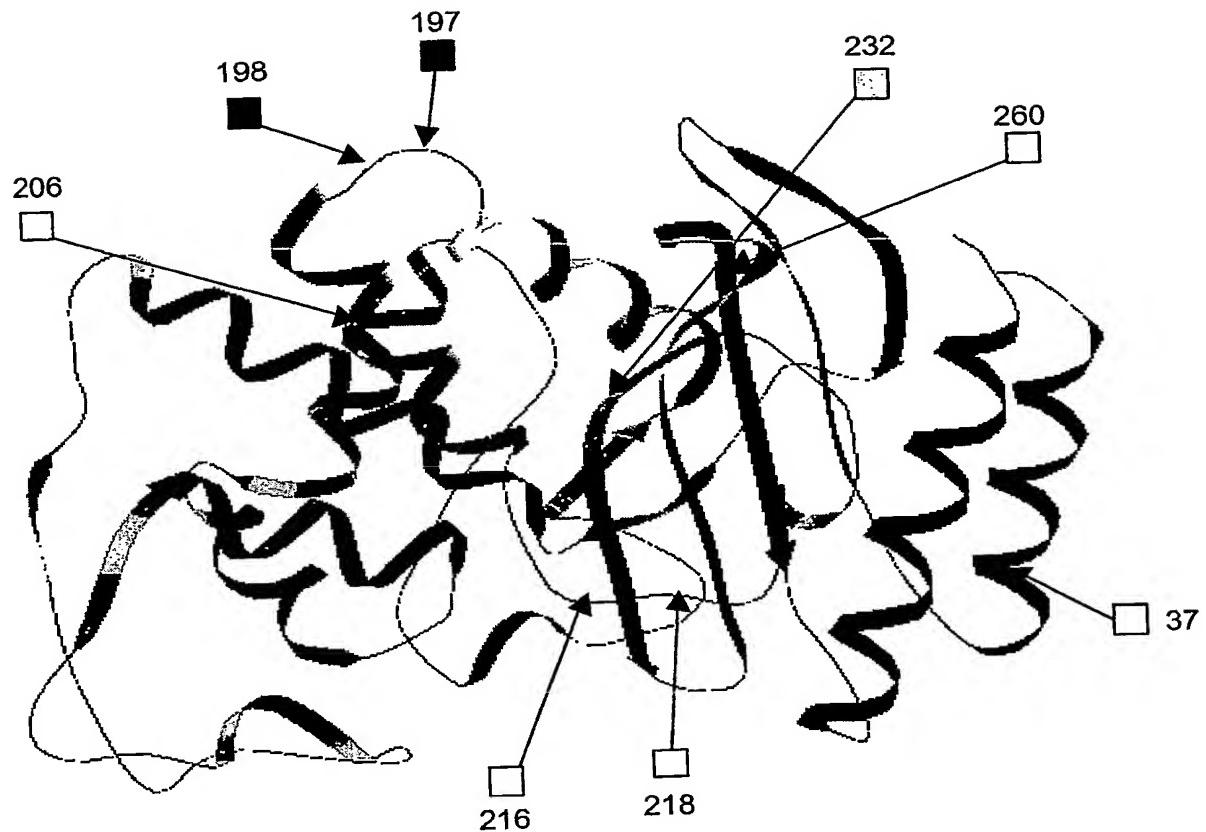


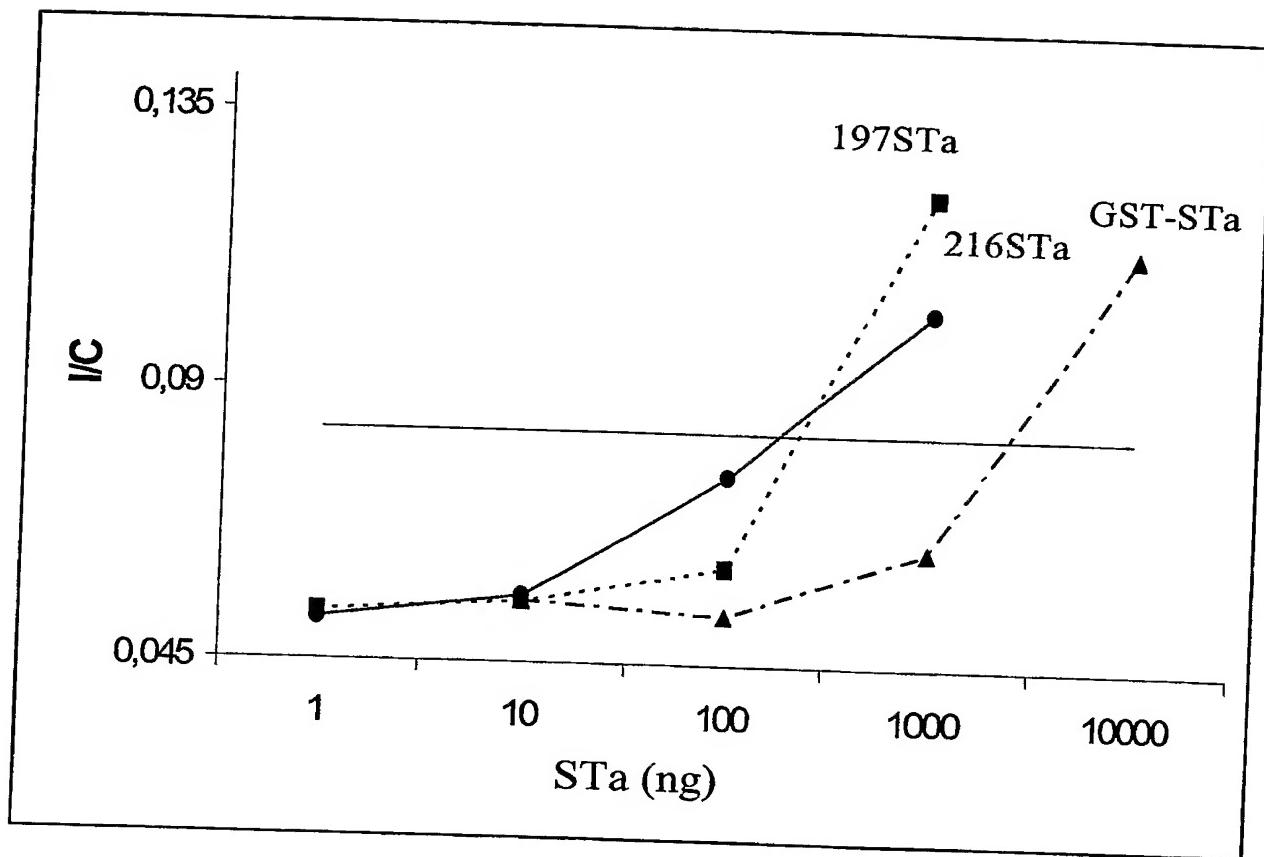
Fig. 7

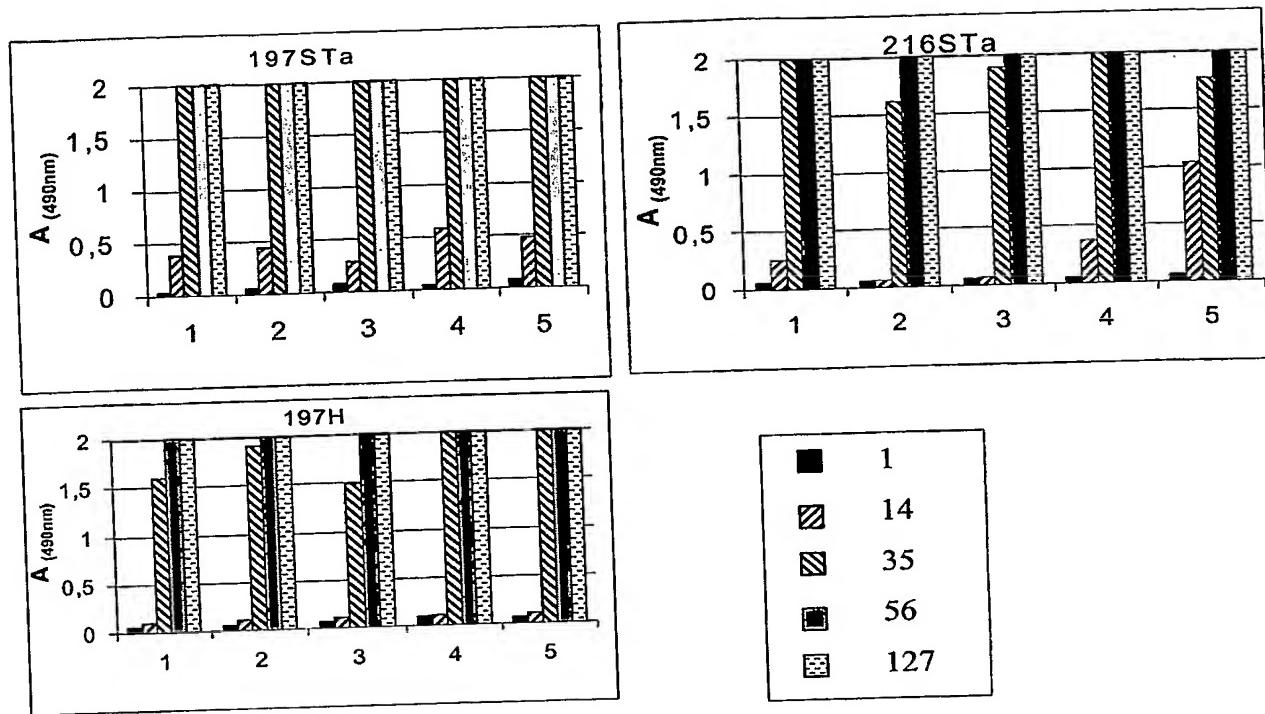
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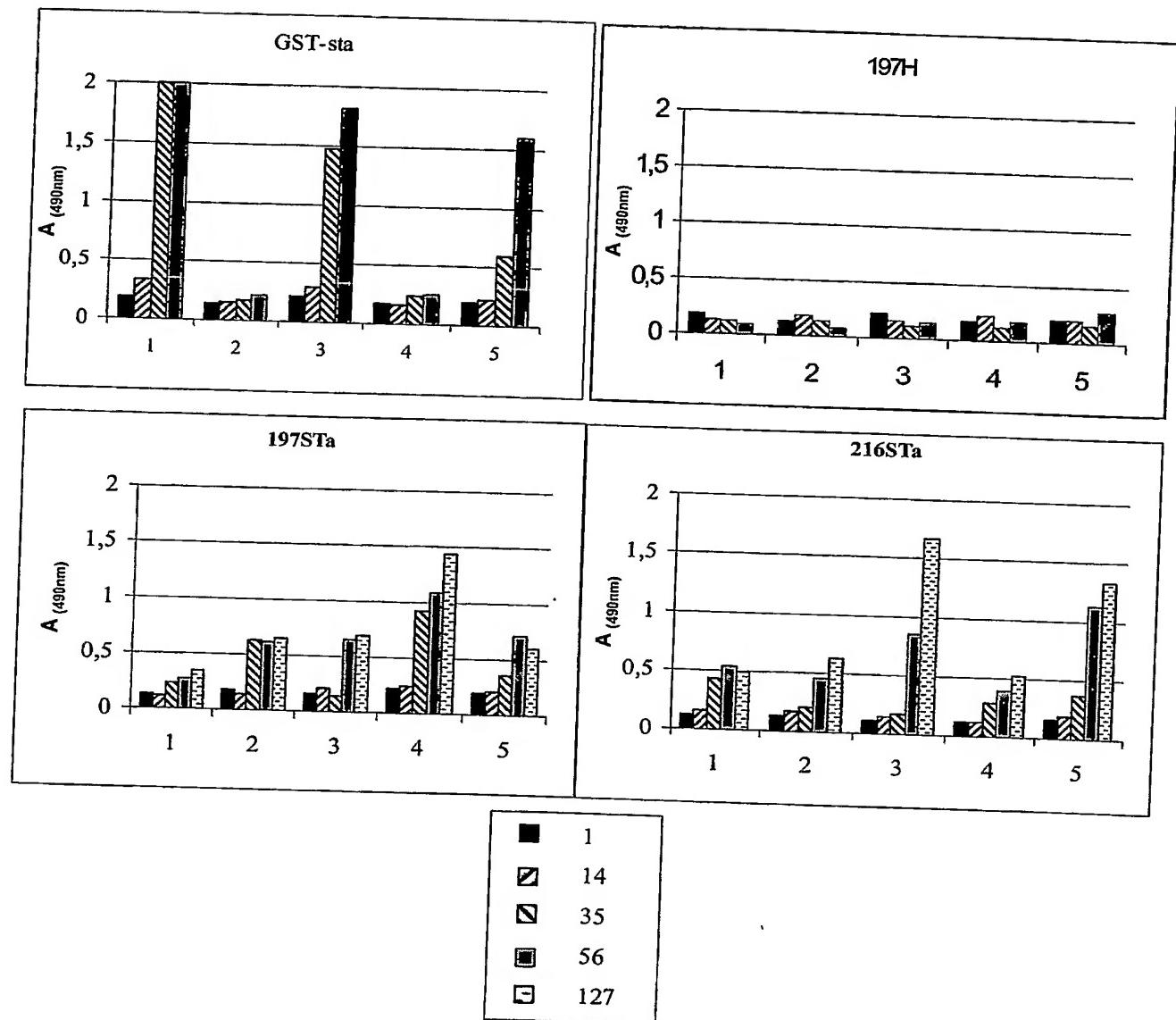
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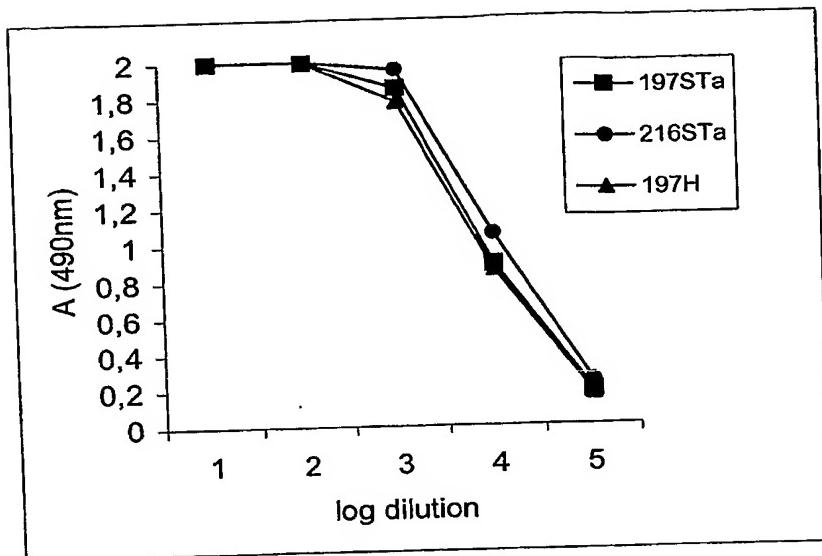
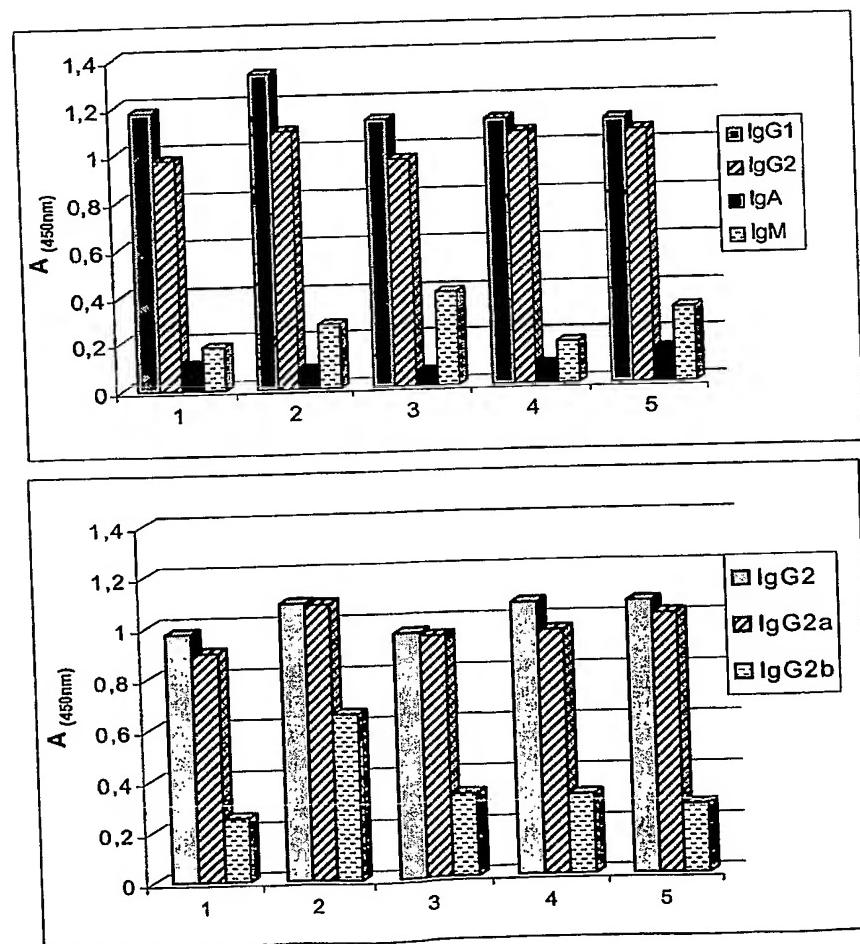
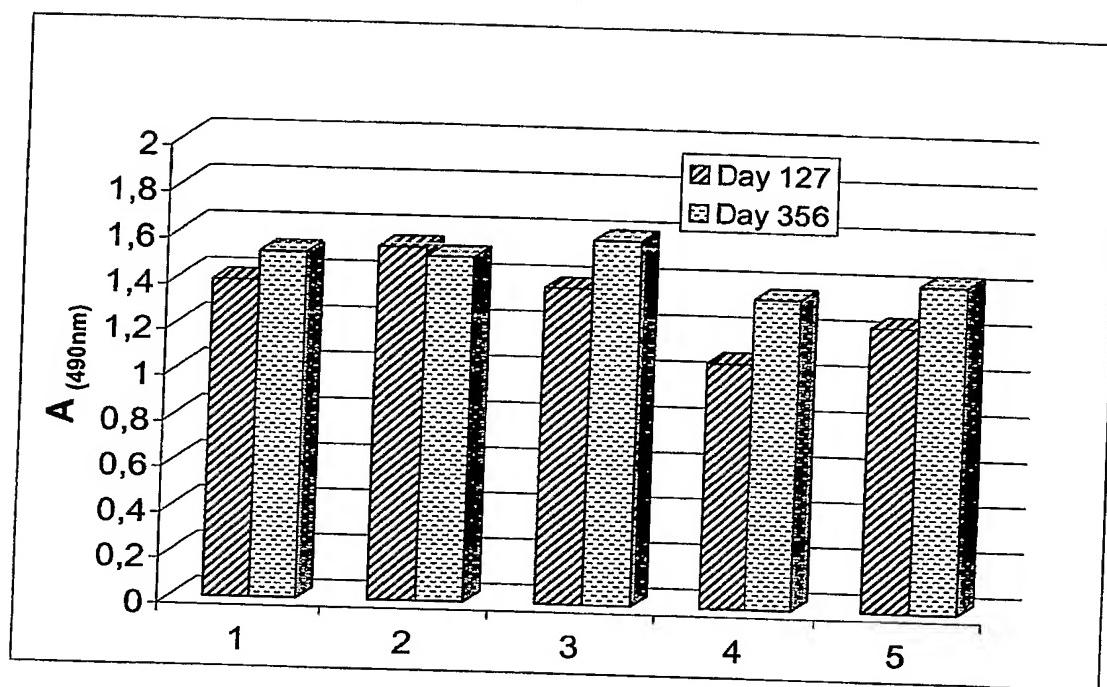
Fig. 9**Fig. 10**

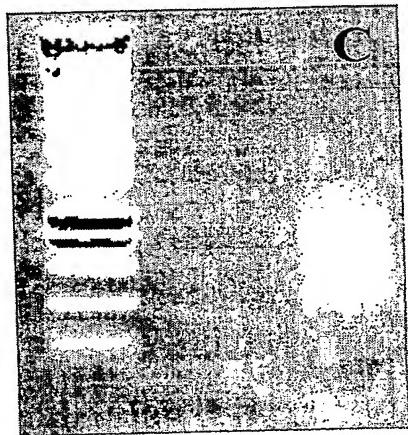
Fig. 11

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Fig. 12 A, B, C

S	E	D	A	B	C	P
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B



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Fig. 12 D, E

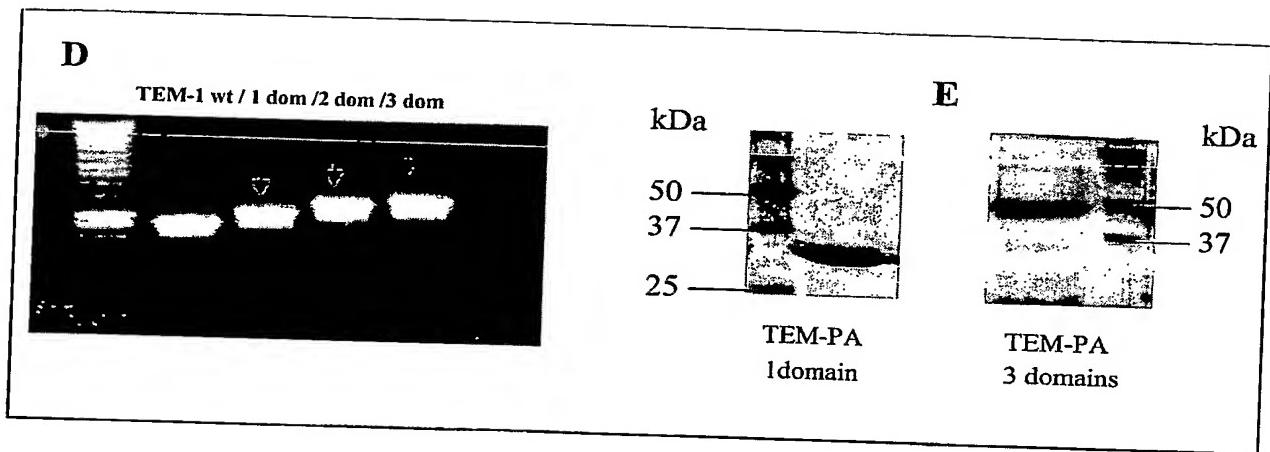


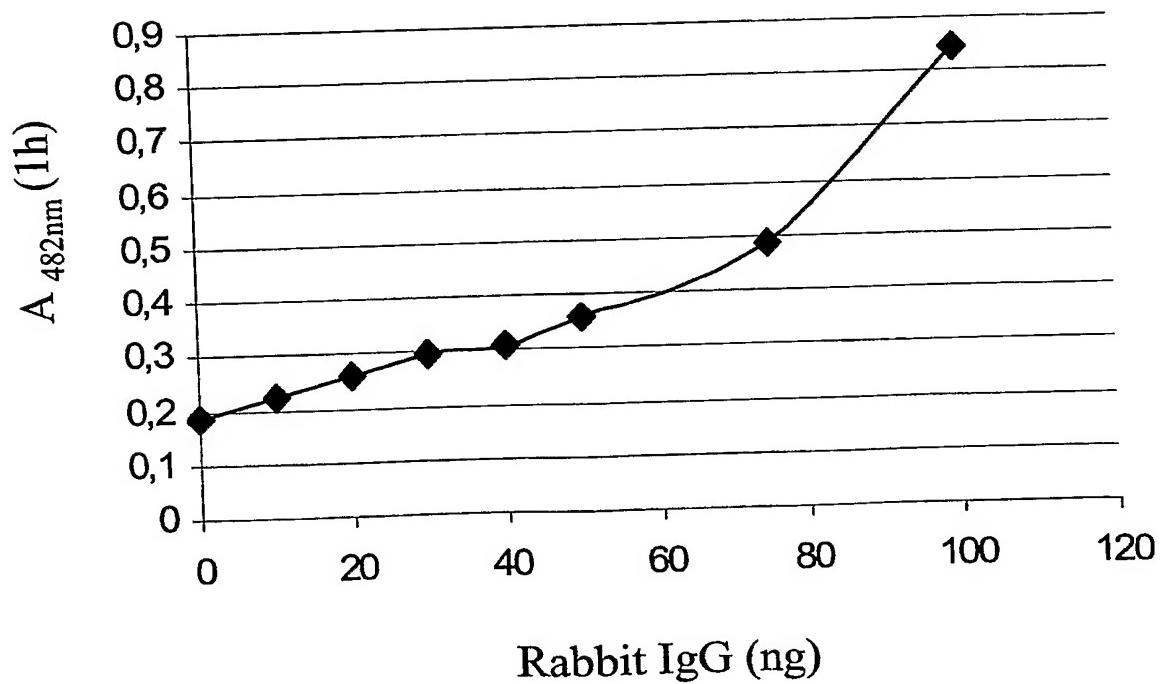
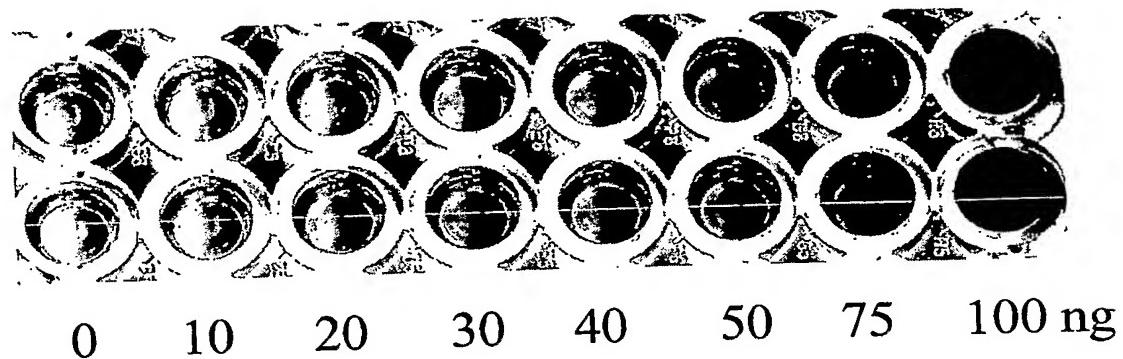
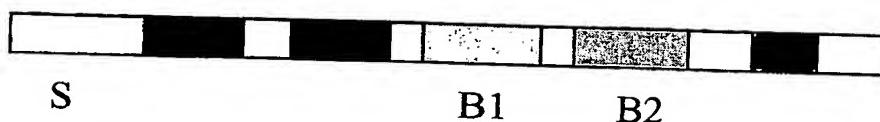
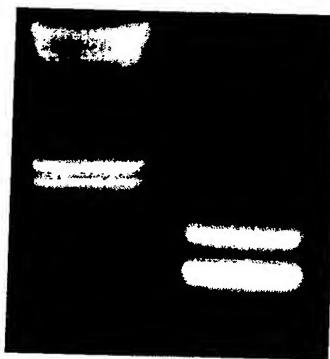
Fig. 13

Figure 14 A B C E

A



C



B



D

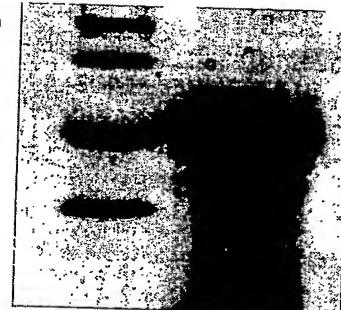


Fig. 15

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Fig. 16

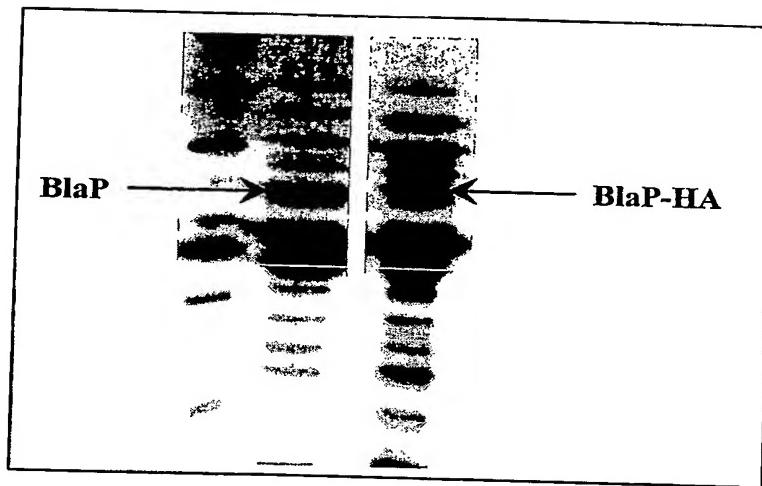
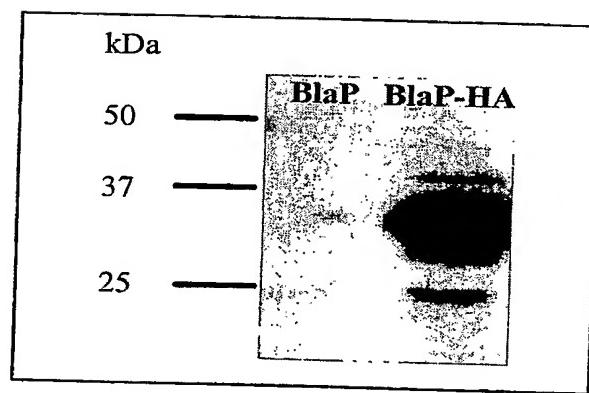
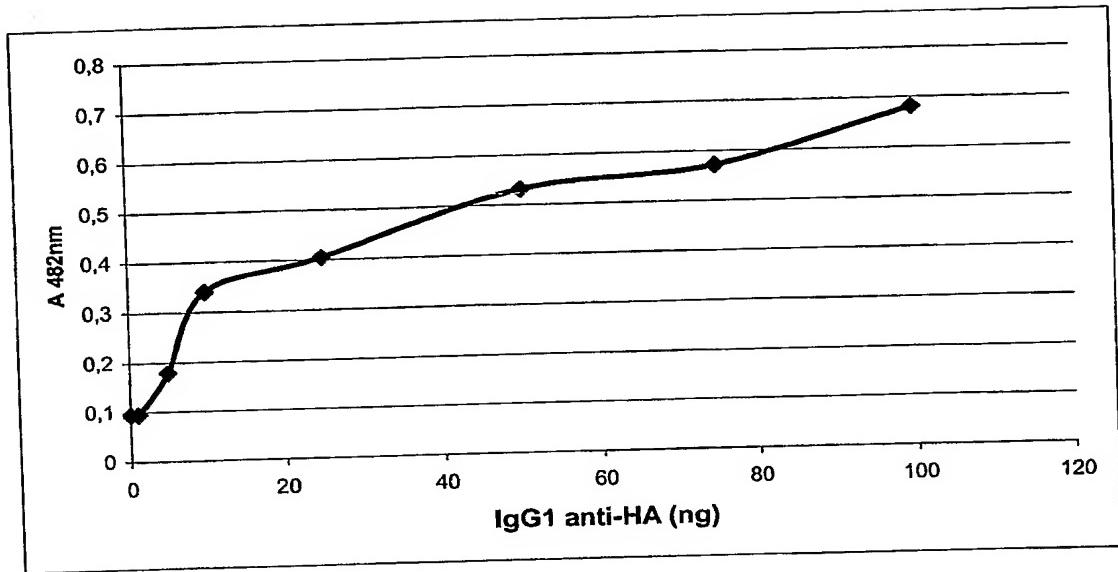


Fig. 17



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Fig. 18



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Fig. 19

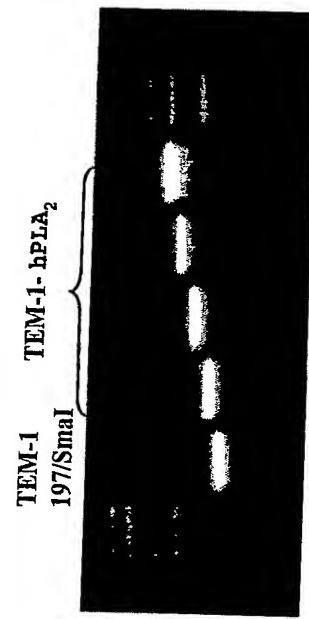
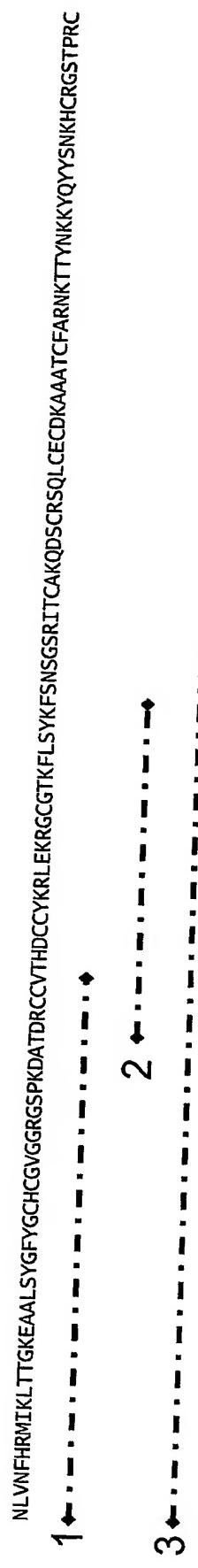
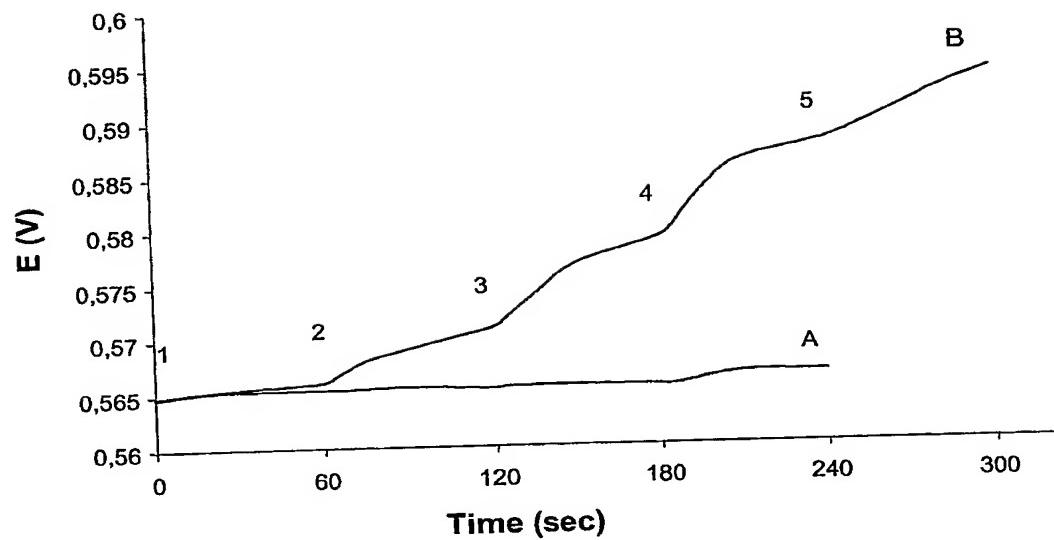


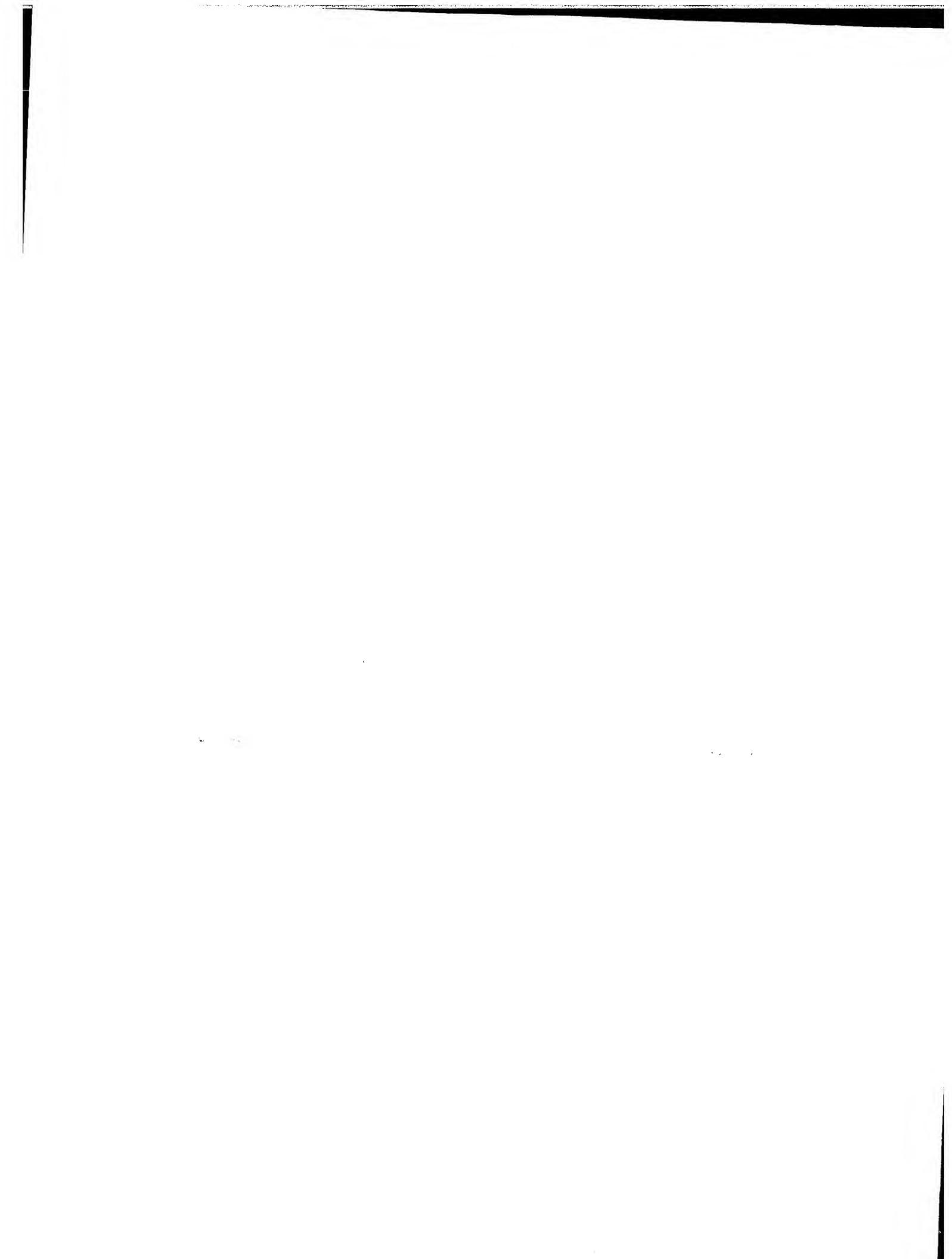
Fig. 20



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Fig. 21





SEQUENCE LISTING

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agtttggaaaa aggaactgag gaagattggatgagttt gatgaggttcaaaatccgaa acgattcgaa 540

ccagagttaa atgaagtgaa tccgggtgaa actcaggata ccagtacagc aagagcactt 600
gtcacaagcc ttcgagcctt tgctcttgaa gataaacttc caagtgaaaa acgcgagctt 660
ttaatcgatt ggatgaaaacg aaataccact ggagacgcct taatccgtgc cggtgtgccg 720
gacggttggg aagtggctga taaaactgga gcggcatcat atggAACCCG gaatgacatt 780
5 gccatcattt ggccgcacaa aggagatcct gtcgtcttg cagtattatc cagcagggat 840
aaaaggacg ccaagtatga tgataaactt attgcagagg caacaaaggt ggtaatgaaa 900
gccttaaaca tgaacggcaa a 921

10 <210> 3
<211> 975
<212> DNA
<213> Streptomyces cacaoi

15 <220>
<223> β -lactamase BlaL gene

<400> 3
atgcgttatcc gtccccacccg tcgtcttctc ctccggcggc tcgcgcgcgt cgccctcggt 60
20 cccgctgggtgg cctgcggtca ggcgtcgccc tccgagagcg gccagcagcc cggcctcgcc 120
ggttgcggga cgagcgcaca cggctcgccg gacgcccacg agaaggagtt cggggcgctg 180
gagaagaagt tcgacgcca ccctggcgctc taegccatcg acacccgcga cggccaggag 240
atcacccacc gggccgacga ggcgttcgccc tacggctcga cttcaaggc cttccaggcg 300
ggcgcgatcc ttgcgcaagt tctccgagac gggcgcgaag tccggcgggg cgccgaggcc 360
25 gacggcatgg acaagggtgtt ccactacggg caggacgcga tcctgcacaa ctcaccgggt 420
accgagaagc acgtcgccga cggcatgtcc ctgcgcgagc tggcgacgc cgtcggtggcc 480
tacagcgaca acacccgcgc caacctgctc ttgcaccagc tcggcgccg aaggggctca 540
acgcgggtcc tcaaggcagct cggcgaccac accacgagca tggaccgcta cgagcaggag 600
ctgggctcgg ccttcggccgt cgaccccccgg gacaccagca cggcgccgc gttcgccgag 660
30 gacctgcgcg ctttcggccgt cgaggacggc gagaaggccg ccctcgccca caacgaccgc 720
gagcagctga acgactggat gagcgggagc aggaccggcg acgcgctgtat cggggccgg 780
gtcccgaaagg actggaaggt ggaggacaag agcggccagg tcaagtacgg caccggaaac 840
gacatcgccg tggccggccc gccccggccgc ggcgcgatcg tcgtctcggt gatgagccac 900
ggcgcacaccc aggacgcccga gccgcacgac gagctggtgg ccgaggccgg ctcgtcgcc 960
35 gccgacggtc tgaag 975

<210> 4
<211> 286
40 <212> PRT
<213> Escherichia coli

<220>
<223> β -lactamase TEM-1

45 <400> 4
Met Ser Ile Gln His Phe Arg Val Ala Leu Ile Pro Phe Phe Ala Ala
1 5 10 15

50 Phe Cys Leu Pro Val Phe Ala His Pro Glu Thr Leu Val Lys Val Lys
20 25 30

Asp Ala Glu Asp Gln Leu Gly Ala Arg Val Gly Tyr Ile Glu Leu Asp
35 40 45

55 Leu Asn Ser Gly Lys Ile Leu Glu Ser Phe Arg Pro Glu Glu Arg Phe
50 55 60

Pro Met Met Ser Thr Phe Lys Val Leu Leu Cys Gly Ala Val Leu Ser

	65	70	75	80
	Arg Val Asp Ala Gly Gln Glu Gln Leu	Gly Arg Arg Ile His Tyr Ser		
	85		90	95
5	Gln Asn Asp Leu Val Glu Tyr Ser Pro Val Thr Glu Lys His Leu Thr			
	100	105	110	
	Asp Gly Met Thr Val Arg Glu Leu Cys Ser Ala Ala Ile Thr Met Ser			
10	115	120	125	
	Asp Asn Thr Ala Ala Asn Leu Leu Leu Thr Thr Ile Gly Gly Pro Lys			
	130	135	140	
15	Glu Leu Thr Ala Phe Leu His Asn Met Gly Asp His Val Thr Arg Leu			
	145	150	155	160
	Asp Arg Trp Glu Pro Glu Leu Asn Glu Ala Ile Pro Asn Asp Glu Arg			
	165	170	175	
20	Asp Thr Thr Met Pro Ala Ala Met Ala Thr Thr Leu Arg Lys Leu Leu			
	180	185	190	
	Thr Gly Glu Leu Leu Thr Leu Ala Ser Arg Gln Gln Leu Ile Asp Trp			
25	195	200	205	
	Met Glu Ala Asp Lys Val Ala Gly Pro Leu Leu Arg Ser Ala Leu Pro			
	210	215	220	
30	Ala Gly Trp Phe Ile Ala Asp Lys Ser Gly Ala Gly Glu Arg Gly Ser			
	225	230	235	240
	Arg Gly Ile Ile Ala Ala Leu Gly Pro Asp Gly Lys Pro Ser Arg Ile			
	245	250	255	
35	Val Val Ile Tyr Thr Thr Gly Ser Gln Ala Thr Met Asp Glu Arg Asn			
	260	265	270	
	Arg Gln Ile Ala Glu Ile Gly Ala Ser Leu Ile Lys His Trp			
40	275	280	285	
	<210> 5			
	<211> 307			
45	<212> PRT			
	<213> Bacillus licheniformis			
	<220>			
	<223> β -lactamase BlaP			
50	<400> 5			
	Met Lys Leu Trp Phe Ser Thr Leu Lys Leu Lys Lys Ala Ala Ala Val			
	1	5	10	15
55	Leu Leu Phe Ser Cys Val Ala Leu Ala Gly Cys Ala Asn Asn Gln Thr			
	20	25	30	
	Asn Ala Ser Gln Pro Ala Glu Lys Asn Glu Lys Thr Glu Met Lys Asp			
	35	40	45	

Asp Phe Ala Lys Leu Glu Glu Gln Phe Asp Ala Lys Leu Gly Ile Phe
50 55 60

5 Ala Leu Asp Thr Gly Thr Asn Arg Thr Val Ala Tyr Arg Pro Asp Glu
65 70 75 80

Arg Phe Ala Phe Ala Ser Thr Ile Lys Ala Leu Thr Val Gly Val Leu
85 90 95

10 Leu Gln Gln Lys Ser Ile Glu Asp Leu Asn Gln Arg Ile Thr Tyr Thr
100 105 110

Arg Asp Asp Leu Val Asn Tyr Asn Pro Ile Thr Glu Lys His Val Asp
15 115 120 125

Thr Gly Met Thr Leu Lys Glu Leu Ala Asp Ala Ser Leu Arg Tyr Ser
130 135 140

20 Asp Asn Ala Ala Gln Asn Leu Ile Leu Lys Gln Ile Gly Gly Pro Glu
145 150 155 160

Ser Leu Lys Lys Glu Leu Arg Lys Ile Gly Asp Glu Val Thr Asn Pro
25 165 170 175

Glu Arg Phe Glu Pro Glu Leu Asn Glu Val Asn Pro Gly Glu Thr Gln
180 185 190

Asp Thr Ser Thr Ala Arg Ala Leu Val Thr Ser Leu Arg Ala Phe Ala
30 195 200 205

Leu Glu Asp Lys Leu Pro Ser Glu Lys Arg Glu Leu Leu Ile Asp Trp
210 215 220

35 Met Lys Arg Asn Thr Thr Gly Asp Ala Leu Ile Arg Ala Gly Val Pro
225 230 235 240

Asp Gly Trp Glu Val Ala Asp Lys Thr Gly Ala Ala Ser Tyr Gly Thr
240 245 250 255

40 Arg Asn Asp Ile Ala Ile Ile Trp Pro Pro Lys Gly Asp Pro Val Val
260 265 270

Leu Ala Val Leu Ser Ser Arg Asp Lys Lys Asp Ala Lys Tyr Asp Asp
45 275 280 285

Lys Leu Ile Ala Glu Ala Thr Lys Val Val Met Lys Ala Leu Asn Met
290 295 300

50 Asn Gly Lys
305

<210> 6
55 <211> 325
<212> PRT
<213> Streptomyces cacaoi

<220>

<223> β -lactamase BlaL

<400> 6
Met Arg Ile Arg Pro Thr Arg Arg Leu Leu Gly Ala Val Ala Pro
5 1 5 10 15
Leu Ala Leu Val Pro Leu Val Ala Cys Gly Gln Ala Ser Gly Ser Glu
20 25 30
10 Ser Gly Gln Gln Pro Gly Leu Gly Gly Cys Gly Thr Ser Ala His Gly
35 40 45
Ser Ala Asp Ala His Glu Lys Glu Phe Arg Ala Leu Glu Lys Lys Phe
50 55 60
15 Asp Ala His Pro Gly Val Tyr Ala Ile Asp Thr Arg Asp Gly Gln Glu
65 70 75 80
Ile Thr His Arg Ala Asp Glu Arg Phe Ala Tyr Gly Ser Thr Phe Lys
20 85 90 95
Ala Leu Gln Ala Gly Ala Ile Leu Ala Gln Val Leu Arg Asp Gly Arg
100 105 110
25 Glu Val Arg Arg Gly Ala Glu Ala Asp Gly Met Asp Lys Val Val His
115 120 125
Tyr Gly Gln Asp Ala Ile Leu Pro Asn Ser Pro Val Thr Glu Lys His
130 135 140
30 Val Ala Asp Gly Met Ser Leu Arg Glu Leu Cys Asp Ala Val Val Ala
145 150 155 160
Tyr Ser Asp Asn Thr Ala Ala Asn Leu Leu Phe Asp Gln Leu Gly Gly
35 165 170 175
Arg Arg Gly Ser Thr Arg Val Leu Lys Gln Leu Gly Asp His Thr Thr
180 185 190
40 Ser Met Asp Arg Tyr Glu Gln Glu Leu Gly Ser Ala Val Pro Gly Asp
195 200 205
Pro Arg Asp Thr Ser Thr Pro Arg Ala Phe Ala Glu Asp Leu Arg Ala
210 215 220
45 Phe Ala Val Glu Asp Gly Glu Lys Ala Ala Leu Ala Pro Asn Asp Arg
225 230 235 240
Glu Gln Leu Asn Asp Trp Met Ser Gly Ser Arg Thr Gly Asp Ala Leu
50 245 250 255
Ile Arg Ala Gly Val Pro Lys Asp Trp Lys Val Glu Asp Lys Ser Gly
260 265 270
55 Gln Val Lys Tyr Gly Thr Arg Asn Asp Ile Ala Val Val Arg Pro Pro
275 280 285
Gly Arg Ala Pro Ile Val Val Ser Val Met Ser His Gly Asp Thr Gln
290 295 300

Asp Ala Glu Pro His Asp Glu Leu Val Ala Glu Ala Gly Leu Val Val
305 310 315 320
5 Ala Asp Gly Leu Lys
325

<210> 7
10 <211> 36
<212> DNA
<213> Artificial Sequence

<220>
15 <223> Description of Artificial Sequence: primer

<400> 7
tcagtttaca atttcaacaa agaacaacaa aatgct 36
20

<210> 8
<211> 30
<212> DNA
<213> Artificial Sequence

25 <220>
<223> Description of Artificial Sequence: primer

<400> 8
30 tcgaaaatttt ttgttgtctt cctcttttgg 30

<210> 9
<211> 33
35 <212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

40 <400> 9
catatgaaaa agaaaaaacat ttattcaatt cgt 33

<210> 10
<211> 33
<212> DNA
<213> Artificial Sequence

45 <220>
<223> Description of Artificial Sequence: primer

<400> 10
50 ggatccttat agttcgcgac gacgtccagc taa 33

<210> 11
<211> 32
<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

5 <400> 11
cgggagctca ggctcaccca gaaacgctgg tg 32

10 <210> 12
<211> 36
<212> DNA
<213> Artificial Sequence

15 <220>
<223> Description of Artificial Sequence: primer

<400> 12
cgggaattct caccaatgct taatcagtga ggcacc 36

20
<210> 13
<211> 39
<212> DNA
25 <213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

30 <400> 13
ggctgtactt acaaatataat ccttaatggc aaaacattg 39

35 <210> 14
<211> 33
<212> DNA
<213> Artificial Sequence

40 <220>
<223> Description of Artificial Sequence: primer

<400> 14
ctctctttca gttaccgtaa aggtcttagt cg 33

45 <210> 15
<211> 39
<212> DNA
<213> Artificial Sequence

50 <220>
<223> Description of Artificial Sequence: primer

<400> 15
aggttttatc catacgacgt cccggactac gccacaact 39

55 <210> 16
<211> 39

<212> DNA
<213> Artificial Sequence

<220>

5 <223> Description of Artificial Sequence: primer

<400> 16
agttgtggcg tagtccggga cgtcgtatgg ataaaacct 39

10 <210> 17
<211> 30
<212> DNA
<213> Artificial Sequence

15 <220>

<223> Description of Artificial Sequence: primer

<400> 17
20 ctcgagaaaa gaaatttggt gaatttccac 30

<210> 18
<211> 30

25 <212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

30 <400> 18
gcaacgtgga gtgctccctc tgcagtgtt 30

35 <210> 19
<211> 90
<212> DNA
<213> Artificial Sequence

40 <220>

<223> Description of Artificial Sequence: primer

<400> 19
ccgatcatca aacttctcaa gctgcttaaa ctcctgcgcc ggaaacttct caagctgctt 60

45 aaactcctgc cgatcagga gtttaagcag 90

<210> 20
<211> 90

50 <212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

55 <400> 20
ctgcttaaac tcctgatccg gcaggagttt aagcagcttg agaagttcc ggcgcaggag 60

tttaagcagc ttgagaagtt tgatgatcgg 90

<210> 21
<211> 54
<212> DNA
5 <213> Escherichia coli

<220>
<223> STa, heat-stable enterotoxin

10 <400> 21 aacacgtttt actgctgcga actttgctgc aaccagcat gcgcaggttg ctac 54

<210> 22
15 <211> 18
<212> PRT
<213> Escherichia coli

<220>
20 <223> STa, heat-stable enterotoxin

<400> 22 Asn Thr Phe Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Ala Gly
1 5 10 15
25 Cys Tyr

30 <210> 23
<211> 201
<212> DNA
<213> Staphylococcus aureus

35 <220>
<223> Protein A, one Fc binding domain

<400> 23 tcagtgaaca atttcaacaa agaacaacaa aatgctttct atgaaatttt acatttacct 60
40 aacttaactg aagaacaacg taacggcttc atccaaagcc taaaagacga tccttcagtg 120
agcaaagaaa ttttagcaga agctaaaaag ctaaacgatg ctcAACGACC AAAAGAGGAA 180
gacaacaaga aaaaatttcg a 201

45 <210> 24
<211> 67
<212> PRT
<213> Staphylococcus aureus

50 <220>
<223> Protein A, one Fc binding domain

<400> 24 Ser Val Asn Asn Phe Asn Lys Glu Gln Gln Asn Ala Phe Tyr Glu Ile
55 1 5 10 15
Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Gly Phe Ile Gln
20 25 30

Ser Leu Lys Asp Asp Pro Ser Val Ser Lys Glu Ile Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys Glu Glu Asp Asn Lys Lys
5 50 55 60

Lys Phe Arg
65

10 <210> 25
<211> 375
<212> DNA
<213> Staphylococcus aureus

15 <220>
<223> Protein A, two Fc binding domains

<400> 25

20 tcagtgaaca atttcaacaa agaacaacaa aatgctttct atgaaaatctt gaacatgcct 60
aacttgaacg aagaacaacg caatggtttc atccaaagct taaaagatga cccaaagtcaa 120
agtgtcaacc tttagcaga agctaaaaag ttaaatgaat ctcaaggcacc gaaagctgat 180
aacaatttca acaaaagaaca acaaaatgct ttctatgaaa tttagcattt acctaactta 240
actgaagaac aacgtAACGG cttcatccaa agccttaaag acgatccttc agtgagcaaa 300
25 gaaatttttag cagaagctaa aaagctaaac gatgctcaag caccaaaaaga ggaagacaac 360
aagaaaaaat ttcga 375

30 <210> 26
<211> 125
<212> PRT
<213> Staphylococcus aureus

35 <220>
<223> Protein A, two Fc binding domains

<400> 26

40 Ser Val Asn Asn Phe Asn Lys Glu Gln Gln Asn Ala Phe Tyr Glu Ile
1 5 10 15

Leu Asn Met Pro Asn Leu Asn Glu Glu Gln Arg Asn Gly Phe Ile Gln
20 25 30

45 Ser Leu Lys Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Glu Ser Gln Ala Pro Lys Ala Asp Asn Asn Phe Asn
50 55 60

50 Lys Glu Gln Gln Asn Ala Phe Tyr Glu Ile Leu His Leu Pro Asn Leu
65 70 75 80

Thr Glu Glu Gln Arg Asn Gly Phe Ile Gln Ser Leu Lys Asp Asp Pro
85 90 95

55 Ser Val Ser Lys Glu Ile Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala
100 105 110

Gln Ala Pro Lys Glu Glu Asp Asn Lys Lys Phe Arg

115

120

125

5 <210> 27
<211> 177
<212> DNA
<213> Streptococcus pyogenes

10 <220>
<223> Protein G, one Fc binding domain

15 <400> 27
ggctgtactt acaaattaat ccttaatggt aaaacattga aaggccaaac aactactgaa 60
gctgttgatg ctgctactgc agaaaaagtc ttcaaacaat acgctaacga caacggtgtt 120
gacggtaat ggacttacga cgatgcgact aagaccttta cggttaactga aagagaa 177

20 <210> 28
<211> 59
<212> PRT
<213> Streptococcus pyogenes

25 <220>
<223> Protein G, one Fc binding domain

30 <400> 28
Gly Cys Thr Tyr Lys Leu Ile Leu Asn Gly Lys Thr Leu Lys Gly Gln
1 5 10 15

35 Thr Thr Thr Glu Ala Val Asp Ala Ala Thr Ala Glu Lys Val Phe Lys
20 25 30

40 Gln Tyr Ala Asn Asp Asn Gly Val Asp Gly Glu Trp Thr Tyr Asp Asp
35 40 45

45 Ala Thr Lys Thr Phe Thr Val Thr Glu Arg Glu
50 55

50 <210> 29
<211> 387
<212> DNA
<213> Streptococcus pyogenes

55 <220>
<223> Protein G, two Fc binding domains

60 <400> 29
ggctgtactt acaaattaat ccttaatggt aaaacattga aaggccaaac aactactgaa 60
gctgttgatg ctgctactgc agaaaaagtc ttcaaacaat acgctaacga caacggtgtt 120
gacggtaat ggacttacga cgatgcgact aagaccttta cggttaactga aaaaaccagaa 180
gtgatcgatg cgtctgaatt aacaccagcc gtgacaactt acaaacttgt tattaatgg 240
aaaacattga aaggcgaaac aactactaa gcagtagacg cagaaactgc agaaaaagcc 300
ttcaaacaat acgctaacga caacggtgtt gatggtgttt ggacttatga tgatgcgact 360
55 aagaccttta cggttaactga aagagag 387

80 <210> 30
<211> 129

<212> PRT

<213> Streptococcus pyogenes

<220>

5 <223> Protein G, two Fc binding domains

<400> 30

Gly Cys Thr Tyr Lys Leu Ile Leu Asn Gly Lys Thr Leu Lys Gly Gln
1 5 10 15

10

Thr Thr Thr Glu Ala Val Asp Ala Ala Thr Ala Glu Lys Val Phe Lys
20 25 30

15

Gln Tyr Ala Asn Asp Asn Gly Val Asp Gly Glu Trp Thr Tyr Asp Asp
35 40 45

Ala Thr Lys Thr Phe Thr Val Thr Glu Lys Pro Glu Val Ile Asp Ala
50 55 60

20

Ser Glu Leu Thr Pro Ala Val Thr Thr Tyr Lys Leu Val Ile Asn Gly
65 70 75 80

Lys Thr Leu Lys Gly Glu Thr Thr Lys Ala Val Asp Ala Glu Thr
85 90 95

25

Ala Glu Lys Ala Phe Lys Gln Tyr Ala Asn Asp Asn Gly Val Asp Gly
100 105 110

30

Val Trp Thr Tyr Asp Asp Ala Thr Lys Thr Phe Thr Val Thr Glu Arg
115 120 125

Glu

35

<210> 31

<211> 39

<212> DNA

<213> Influenza virus

40

<220>

<223> hemagglutinin epitope

<400> 31

45 aggttttatac catacgacgt cccggactac gccacaact

39

<210> 32

<211> 13

50 <212> PRT

<213> Influenza virus

<220>

<223> Hemagglutinin epitope

55

<400> 32

Arg Phe Tyr Pro Tyr Asp Val Pro Asp Tyr Ala Thr Thr
1 5 10

<210> 33
<211> 384
<212> DNA
5 <213> Homo sapiens

<220>
<223> phospholipase (hPLA2)

10 <400> 33
ctcgagaaaa gaaatttgggt gaatttccac agaatgatca agttgacgac aggaaaggaa 60
gccgcactca gttatggctt ctacggctgc cactgtggcg tgggtggcag aggatcccc 120
aaggatgcaa cgatcgctg ctgtgtcaact catgactgtt gctacaaaacg tctggagaaa 180
cgtggatgtg gcacccaaatt tctgagctac aagtttagca actcggggag cagaatcacc 240
15 tggcaaaac aggactcctg cagaagtcaa ctgtgtgagt gtgataaggc tgctgccacc 300
tggggctta gaaacaagac gacctacaat aaaaagtacc agtactattc caataaacac 360
tgagggga gcactccacg ttgc 384

20 <210> 34
<211> 128
<212> PRT
<213> Homo sapiens

25 <220>
<223> phospholipase (hPLA2)

<400> 34
Leu Glu Lys Arg Asn Leu Val Asn Phe His Arg Met Ile Lys Leu Thr 15
30 1 5 10 15

Thr Gly Lys Glu Ala Ala Leu Ser Tyr Gly Phe Tyr Gly Cys His Cys 30
20 25 30

35 Gly Val Gly Arg Gly Ser Pro Lys Asp Ala Thr Asp Arg Cys Cys 45
35 40 45

Val Thr His Asp Cys Cys Tyr Lys Arg Leu Glu Lys Arg Gly Cys Gly 50
50 55 60

40 Thr Lys Phe Leu Ser Tyr Lys Phe Ser Asn Ser Gly Ser Arg Ile Thr 80
65 70 75 80

Cys Ala Lys Gln Asp Ser Cys Arg Ser Gln Leu Cys Glu Cys Asp Lys 45
45 85 90 95

Ala Ala Ala Thr Cys Phe Ala Arg Asn Lys Thr Thr Tyr Asn Lys Lys 100
100 105 110

50 Tyr Gln Tyr Tyr Ser Asn Lys His Cys Arg Gly Ser Thr Pro Arg Cys 125
115 120 125

55

<210> 35
<211> 90
<212> DNA

<213> Artificial Sequence
<220>
5 <223> Description of Artificial Sequence: affinity to
LPS

<400> 35
ccgatcatca aacttctcaa gctgcttaaa ctcctgcgcc ggaaaacttct caagctgctt 60
aaactcctgc cggatcagga gtttaagcag 90
10

<210> 36
<211> 30
<212> PRT
15 <213> Artificial Sequence

<220>
20 <223> Description of Artificial Sequence: affinity to
LPS

<400> 36
Pro Ile Ile Lys Leu Leu Lys Leu Leu Lys Leu Leu Arg Arg Lys Leu
1 5 10 15

25 Leu Lys Leu Leu Lys Leu Leu Pro Asp Gln Glu Phe Lys Gln
20 25 30